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PHYSIOLOGICAL ZOÖLOGY

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ANALYSIS OF SOME PHASES OF MELANOBLAST MIGRATION IN THE BARRED PLYMOUTH ROCK EMBRYOS[1]

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Department of Zoölogy, University of California, Berkeley

IT HAS been established that melanophores in birds, as well as in most other vertebrates, originate from the neural crest (see reviews by DuShane, 1943, 1944). Melanoblasts then migrate away from the vicinity of the neural tube to various regions of the body. Scattered information is available in the literature concerning the time at which these cells arrive at certain destinations, such as the epidermis of the wing bud or leg bud (Willier and Rawles, 1940; Watterson, 1942). Up to the present, however, no definite schedule has been established which gives the time of departure of melanoblasts from different anteroposterior levels of the neural crest and the time of arrival of these cells at various regions of the body. The present study proposes to establish such a schedule for one breed of fowl, the Barred Plymouth Rock. Once such information is available, the whereabouts of melanoblasts at any stage of development can be correlated with other developmental changes in their immediate vicinity, and it might then be possible to determine whether there are any localized differentials along the main axis of the body which influence the time of onset and the direction of melanoblast migration.

At present, the only factor experimentally shown to affect the migration of the melanoblasts is a mutual repulsion of the cells which causes them to move apart (Twitty, 1944, 1945; Twitty and Bodenstein, 1939, 1944). Most of the observations will refer to the migration of those melanoblasts which ultimately enter the epidermis and produce the pigment responsible for the coloration of the down feathers. However, some observations, although less complete, will also be made concerning the time of arrival of melanoblasts in certain of the mesodermal derivatives, such as gonads and mesonephros. As a background for these latter observations, the distribution of visible melanophores throughout the interior of the body of Barred Plymouth Rock embryos at various developmental stages has been recorded.

MATERIALS AND METHODS

Melanoblasts during their migratory phase cannot be identified with certainty

[1] Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of California, Berkeley. I should like to express my gratitude for the Abraham Rosenberg Research Fellowship, which was held for two years, during which time the major part of this study was completed. In addition, I wish to acknowledge my very deep indebtedness to Dr. R. L. Watterson both for suggesting this problem and for his interest and advice throughout the investigation.

in histological preparations (Watterson, 1942). However, their presence or absence in any tissue or organ of the body at any stage of development is readily ascertained by an experimental test (Willier and Rawles, 1940). If a given region of the body, be it epidermis, mesonephros, gonad, etc., is taken from a Barred Plymouth Rock donor embryo and transplanted to the base of the wing bud of a White Leghorn embryo, it can later be identified in the host as a small mass embedded in the base of the wing bud or within the coelom attached to the body wall. If the transplanted tissue or organ contained melanoblasts at the time of the operation, melanophores will appear in the graft or will invade adjacent host tissues and feather germs. That part of the body of the donor from which such a graft was taken may therefore be recorded as positive for the presence of melanoblasts. If the implant does not yet contain melanoblasts at the time of the operation, it may differentiate, but no melanophores will appear in the graft. The region of the donor from which such a graft was taken may be recorded as negative for the presence of melanoblasts. In many instances no trace of the implant can be found at all. Such cases are best discarded, since there is no way of knowing whether the transplant which has failed to persist contained melanoblasts or not; only those cases in which the transplant can be identified but in which no melanophores differentiate can be classified as true negatives.

By systematically applying this experimental test for the presence or absence of melanoblasts to various regions of the body at various stages of development, it was possible to follow the migration of the prospective pigment cells in considerable detail, and the general pattern of the spread of melanoblasts throughout the body became evident. A total of 2,147 transplantations were made. After discarding those hosts which died before melanoblasts could differentiate into melanophores, as well as all hosts in which the grafts could not be identified with certainty, there remained a total of 1,133 transplantations upon which this study is based.

The Barred Plymouth Rock donor eggs were obtained, for the most part, from the Dryden Poultry Farm, Modesto, California; the White Leghorn host eggs were obtained from the Poultry Division of the University of California in Berkeley. It was found expedient to store the unincubated eggs at 12° C. until needed, for both fertility and survival of operated embryos were impaired when the eggs were stored at room temperature. The records show that storage of the unincubated eggs at room temperature resulted in 43 per cent survival of host embryos, whereas storage at 12° C. resulted in 82 per cent survival of host embryos. All eggs were returned to room temperature for 4 or 5 hours before incubation. The incubators were without a forced draft and were kept at 103° ± 2° F.

The transplantation technique used in this study was essentially that described by Rawles (1939) and Hamburger (1942). After the position of the White Leghorn host was ascertained by candling, the eggs were placed on cotton nests in Syracuse watch glasses, where they remained until the operation was completed. A small rectangular area of the shell overlying the embryo was outlined with a hacksaw blade and lifted off. Part of the shell membranes, thus uncovered, were removed with jeweler's forceps. This procedure exposed all membranes inclosing the embryo. These were lightly stained by touching them with a slender glass rod which had first been tipped with agar and subsequently impregnated with Nile blue sulphate. Then the membranes were

slit with a fine glass needle above the region of the wing bud. Vital stain was next applied to this area of the host, the rectangle of shell was replaced, and the host temporarily returned to the incubator. Embryos of stage 4 (Hamburger, 1939), in which the wing bud was just forming, were found to be the most successful hosts, although fairly advanced wing-bud stages (stages 5 and 6) also proved satisfactory. The increased vascularity of the wing bud in the older hosts made the operation more difficult because hemorrhages were often fatal. Four hosts were prepared at one time and returned to the incubator while grafts were isolated from a donor.

Barred Plymouth Rock donors ranged in age from 6 to more than 50 somites. They were exposed by chipping away a fairly large part of the shell and removing both the shell membranes and the embryonic membranes. The entire embryo was then stained with Nile blue sulphate, and the egg was returned to the incubator for a few minutes to allow the stain to penetrate. The blastoderm was then cut out with fine scissors and transferred to a dish of 0.9 per cent NaCl solution. After the number of somites was counted, the tissue or organ to be transplanted was isolated either with fine glass needles or with small razor-blade knives (Burch, 1942). Usually four grafts were removed from a donor, and each was placed in an individual stender dish of warm saline solution and kept at 103° F. on a small thermostatically controlled warming plate until needed. Each graft was then transferred with a pipette to a host in a drop of saline and manipulated with a blunt glass needle into a small slit which had previously been made in the base of the wing bud of the host. Occasionally a graft slipped through into the coelom, instead of remaining in the base of the wing. Specific details of the tissues isolated and the method of isolation will be presented in appropriate sections of these observations.

After the operation, the piece of shell which had been removed in order to expose the host embryo was replaced and sealed with melted paraffin. The eggs were then removed from the cotton nests and placed in trays in the incubator, where they were allowed to develop undisturbed for an additional 10 or 11 days. They were then opened and examined for the presence or absence of pigmentation.

Observations

I. Migration of melanoblasts into the epidermis

The first group of experiments was designed to ascertain the time of arrival of melanoblasts in various regions of the epidermis of the developing embryo. Figure 1 indicates representative epidermal regions of the donor, which were tested for the presence or absence of melanoblasts. In order to determine the time of departure of melanoblasts from the neural tube, pieces of epidermis were cut out just lateral to the neural tube, either overlying the somites or anterior or posterior to the somites (see grafts labeled *a* in Fig. 1). Grafts removed from these regions constitute series A. Series B is concerned with the time of the appearance of the prospective pigment cells in the epidermis lateral to the somites, including that covering the basal portion of the limb buds and the gill arches (see grafts labeled *b* in Fig. 1). Series C consists of a more detailed analysis of the spread of melanoblasts into the epidermis within the limb buds (see grafts labeled *c* in Fig. 1).

Fine glass needles were used to excise all epidermal grafts. Four cuts were made outlining the area to be grafted; then the needle was run beneath the

piece of tissue, separating it from the underlying mesoderm. Although every attempt was made to include as little mesenchyme in the graft as possible, it is almost certain that the epidermis carried over some mesenchyme cells with it. The epidermis is extremely thin and clings to the underlying mesoderm (particularly in the somite region) frequently ripping upon removal. Consequently, it proved impracticable to try to obtain pieces of a standard size, although most transplants covered the length of approximately five somites.

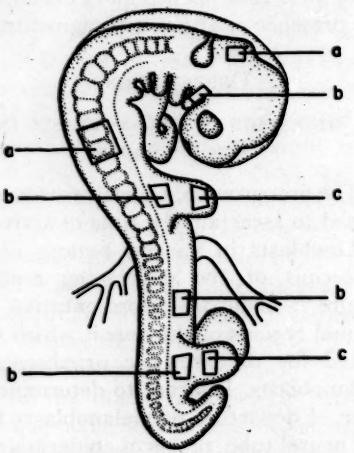


FIG. 1.—Embryo, showing representative regions from which epidermal grafts were taken. Grafts marked *a* are lateral to the neural tube; grafts marked *b* are lateral to the somites or in the gill-arch region; grafts marked *c* are regional grafts within the limb buds.

A. INTO EPIDERMIS LATERAL TO NEURAL TUBE

The observations on this series of transplants are presented in Figure 2. This method of recording the data has been used, since it tabulates the results of each separate transplant and, in addition, even if the figure is not examined in detail, gives a visual picture of the time of onset of melanoblast migration at different anteroposterior levels of the body. The age of the donor in somites is recorded on the abscissa, and the body-levels covered by the epidermis which was tested are indicated on the ordinate. Each vertical line represents a single transplant; solid lines indicate the presence of melanoblasts in a graft; broken lines indicate the absence of melanoblasts. It should be emphasized that a solid line indicates only that melanoblasts are present in some part of the graft, not necessarily throughout its full extent; this difficulty, however, is minimized by the existence of many overlapping grafts. For example, in 17- and 18-somite donors the grafts covering somite levels 1–4 or 1–5 are positive for the presence of melanoblasts. However, other grafts covering somite levels 3–6 are negative, suggesting that at this stage melanoblasts are lateral to the neural tube only over somites 1 and 2.

The narrow, solid, horizontal lines indicate the posterior limits of the last-formed pair of somites and are placed there for convenience, helping to emphasize the relation between the body-level at which migration of melanoblasts is under way and the level of the last somite. Below these lines are recorded the results of epidermal grafts removed from the region covering the unsegmented mesodermal bands (segmental plates). No attempt has been made to draw these latter lines to exact scale, but grafts extending from the border of the last somite to the tail bud or Hensen's node are represented as extending the length of two somites; grafts covering only a part of this distance are represented by shorter bars.

As can be seen in Figure 2, the melanoblasts first spread laterally from the neural tube at the level of the mesencephalon in the 8- to 10-somite chicks. Melanoblasts were definitely beginning

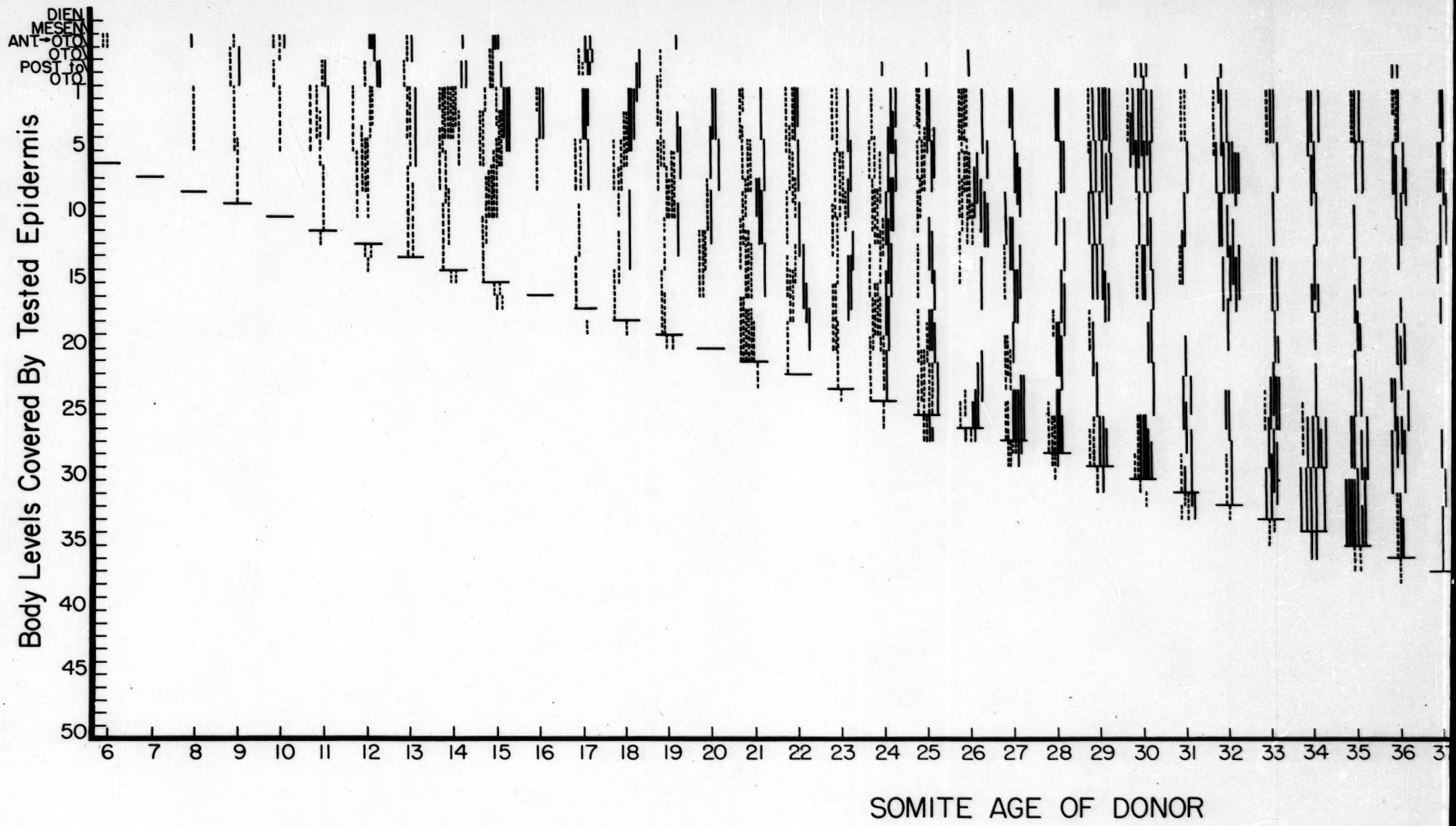
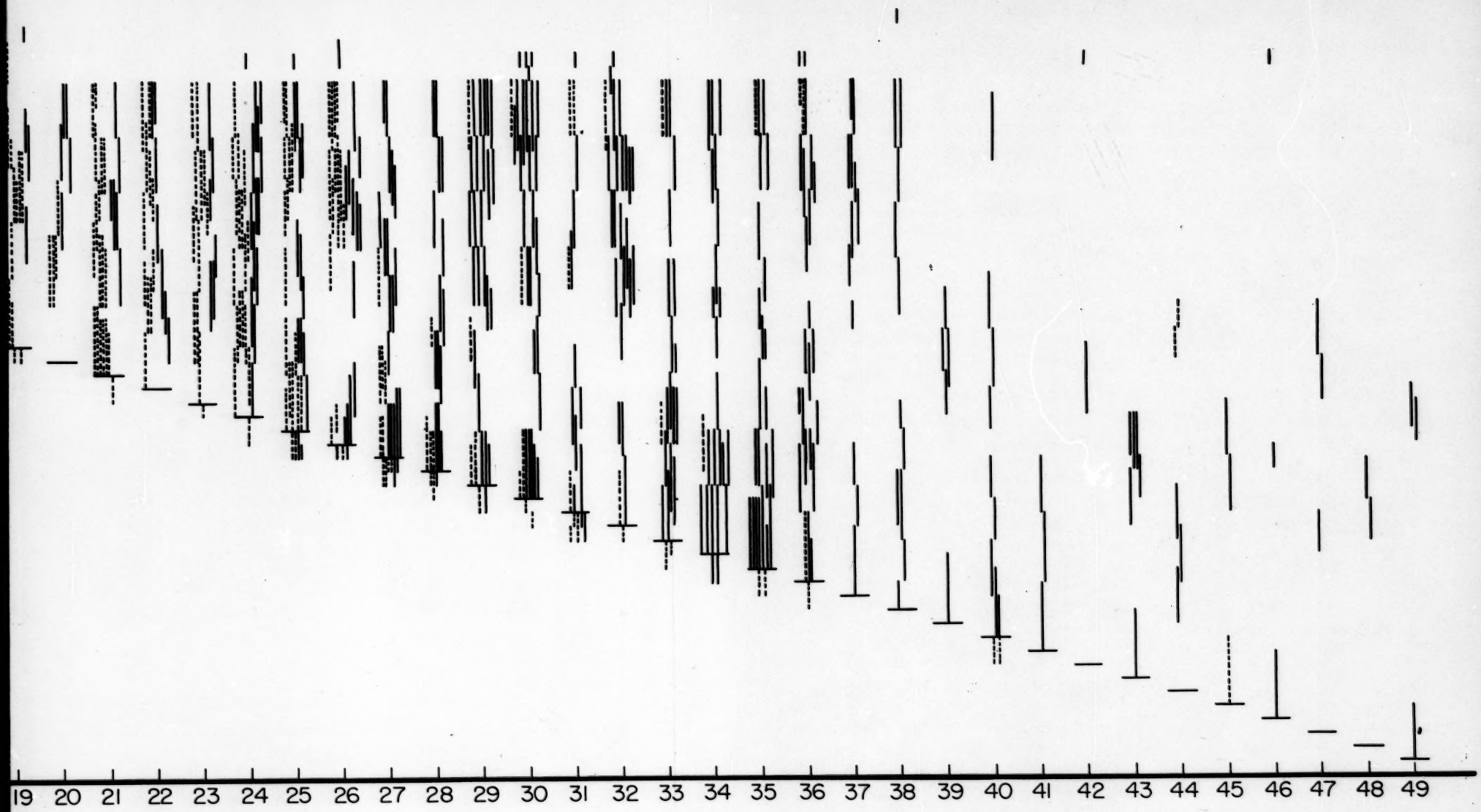


FIG. 2.—Migration of melanoblasts into the epidermis lateral to the neural tube. Solid lines represent grafts yielding melanophores; broken lines represent grafts not yielding melanophores. *Dien.* = Diencephalon; *Mesen.* =



id lines represent grafts yielding melanophores; broken lines represent grafts not yielding melanophores. *Dien.* = Diencephalon; *Mesen.* = Mesencephalon; *Oto.* = Otocyst. For further explanation see text.

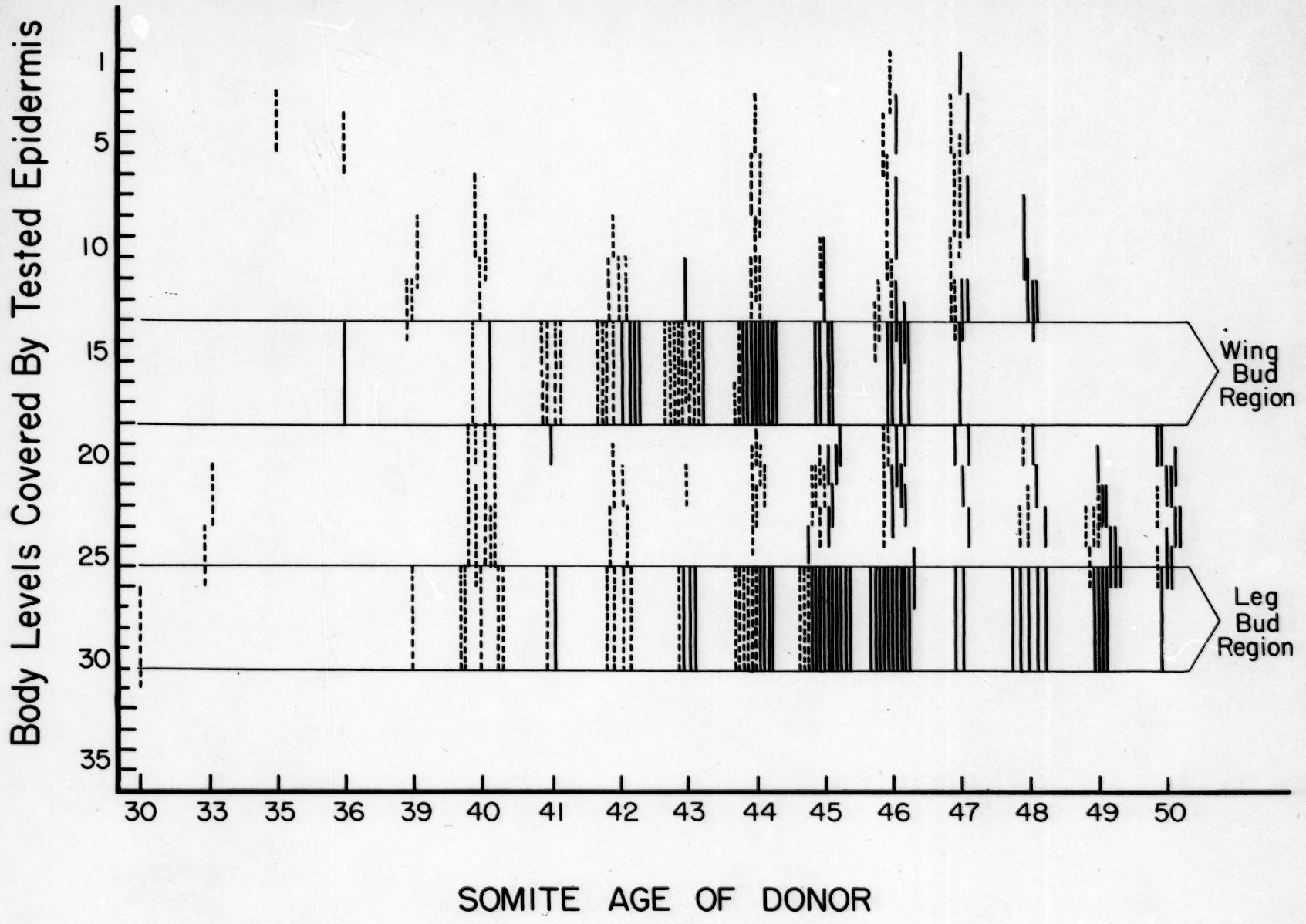
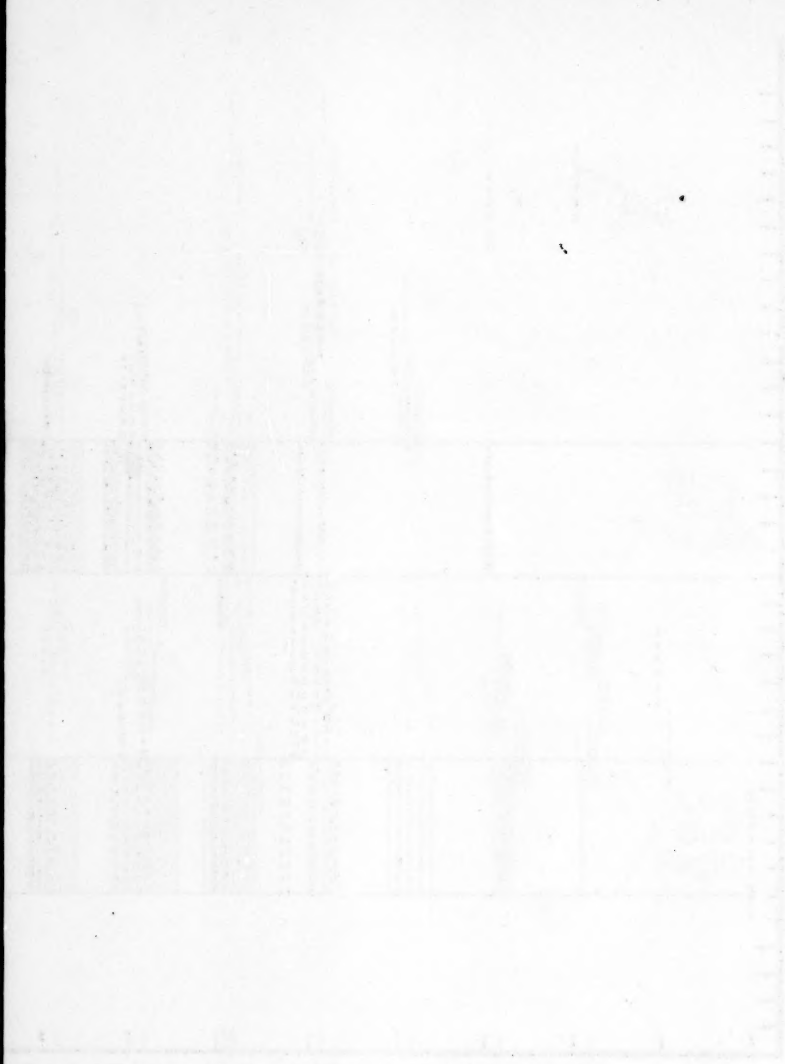


FIG. 3.—Migration of melanoblasts into the epidermis lateral to the somites. Solid lines represent grafts yielding melanophores; broken lines represent grafts not yielding melanophores. Note the precocious appearance of positive grafts in the regions of the limb buds.



to appear in association with epidermis overlying the first few somites by the time of the 15-somite stage. In 15- to 20-somite embryos, melanoblasts had not yet departed from more posterior levels of the neural crest, aside from a few sporadic instances.

From somite stage 21 to stage 26 the picture of melanoblast migration was less consistent (Fig. 2). Both positive and negative grafts were obtained in almost equal numbers from epidermis overlying all somites except the last five, including the level of the first five somites which had previously been almost uniformly positive. Since the transplants from these stages were so inconsistent, the data are presented in greater detail in Table 1, in order to show which grafts came from a single donor. Sometimes embryos of the same somite age gave markedly different results; for example, in one 26-somite donor (No. 75) the epidermis covering somites 6–12 contained no melanoblasts, whereas in another 26-somite embryo (No. 79) this same region contained melanoblasts. Even within a single donor embryo the outward migration of melanoblasts apparently failed to follow a strict anteroposterior sequence, although such a sequence seemed to be followed in earlier stages (witness embryos Nos. 30, 44, and 74). As a rule, grafts were taken only from the right side of the donor, but in a few cases grafts from the same somite levels were removed from both sides of an embryo. Here, too, the results were conflicting. In embryos Nos. 14, 60, and 70, bilateral grafts seemed to indicate a symmetrical migration of melanoblasts, whereas embryos Nos. 18 and 25 suggested a bilateral asymmetry. However, it must be kept in mind that slight differences in the position of the isolating cut nearest to the midline may be the deciding factor between a positive and a negative test in each individual operation. No single operation is conclusive. At best, only the over-all picture given by all operations approaches an accurate representation of the outward spread of the melanoblasts.

Beyond the 27-somite stage (Fig. 2) the epidermis overlying all the somites tended to yield melanophores upon transplantation, except for a few sporadic negative grafts chiefly from the regions of the first and last five somites. During all early stages the most posterior level at which melanoblast migration was beginning was always considerably anterior to the most posterior somite; it was only after the 30-somite stage that the onset of migration of melanoblasts consistently occurred as far posteriorly as the last somite. Thus at all earlier stages there appears to be a considerable lag between the formation of additional somites and the onset of melanoblast migration out over these somites, whereas later these two events occur simultaneously. The epidermis lying above the segmental plate rarely contained melanoblasts.

B. INTO EPIDERMIS LATERAL TO SOMITES

Although series A indicated a rough anteroposterior sequence in the departure of melanoblasts from the neural tube and in their arrival beneath or within the epidermis immediately lateral to the central nervous system, their continued migration into more lateral areas did not follow the same sequence. As Figure 3 (constructed in the same way as Fig. 2) shows, the melanoblasts first reached the epidermis lateral to the somites at the level of somites 14–18, which is the region of the base of the wing bud. This region definitely became populated with melanoblasts by the 44-somite stage, and some reached it even earlier. Shortly thereafter melanoblasts also entered the epidermis lateral to somites 26–30, which is the level of the leg bud, and

TABLE 1

MIGRATION OF MELANOBLASTS INTO EPIDERMIS LATERAL TO THE NEURAL TUBE OF 20- TO 26-SOMITE EMBRYOS

Stage	Embryo	Graft	Somite Level	Result
20-somite	1	816	8–11	−
		814	12–16	−
	2	873	12–15	−
	3	988	1– 4	+
		989	4– 7	+
	4	1,013	1– 4	+
		1,015	9–12	+
	5	1,083	5– 8	+
		1,084	12–14	−
21-somite	6	728	17–21	−
	7	890	11–14	−
		889	18–21	−
		888	19–21	−
	8	891	18–21	−
		892	Unseg.	−
	9	843	18–21	−
	10	849	17–21	−
	11	984	4– 7	−
	12	1,039	5– 8	−
	13	1,041	1– 2	−
		1,043	8–10	−
	14	1,196	12–16	−
		1,197	12–16	−
	15	1,365	5– 8	+
	16	1,550	9–12	−
	17	1,564	5– 8	−
		1,565	9–12	+
		1,563	13–16	−
	18	1,569	1– 4	−
		1,567	1– 4	+
		1,568	8–10	+
	19	1,573	8–12	+
		1,576	13–16	+
22-somite	20	811	4– 8	−
		812	14–16	−
	21	722	10–13	+
		723	14–17	+
		721	18–20	+
		720	21–22	−
	22	996	6– 9	−
		997	15–18	−
	23	1,099	1– 3	+
		1,098	8–10	−
		1,100	12–15	−
		1,097	19–22	−?
	24	1,140	5– 8	+
		1,141	9–12	−
		1,142	13–15	−
	25	1,262	1– 4	−
		1,263	1– 4	+
	26	1,288	1– 5	−
		1,291	16–18	+
23-somite	27	206	8–10	+
	28	740	6– 9	−
23-somite *cont.*		739	10–13	−
		738	14–18	+
	29	1,018	Unseg.	−
	30	1,113	1– 4	−
		1,115	5– 8	+
		1,112	9–11	−
		1,114	12–14	+
	31	1,153	1– 5	+
		1,155	6–10	−
		1,154	16–20	−
	32	1,159	1– 5	−
		1,160	6–10	−
		1,161	11–15	−
		1,158	16–20	−
	33	1,294	19–23	−
	34	1,328	14–17	+
24-somite	35	146	17–21	−
		145	21–24	+
	36	161	Unseg.	−
	37	274	15–19	+
		273	20–23	−
	38	808	11–13	−
	39	967	9–12	−
		966	17–20	−
	40	977	8–11	−
		978	16–19	−
		976	20–24	−
	41	1,006	9–12	+
		1,005	13–16	−
		1,007	17–20	+
	42	1,022	1– 5	+
		1,025	11–15	+
		1,023	17–20	+
	43	1,030	13–16	−
	44	1,108	1– 4	−
		1,111	5– 8	+
		1,110	9–12	−
		1,109	13–16	+
	45	1,171	5– 7	−
		1,172	8–11	+
		1,169	12–15	−
		1,170	16–19	−
	46	1,177	1– 3	+
		1,179	4– 8	+
		1,176	9–12	+
	47	1,184	5– 7	−
		1,186	8–11	+
	48	1,301	3– 5	+
	49	1,321	6– 9	−
25-somite	50	20	11–14	+
		21	15–17	+
	51	22	17–20	+
		23	21–25	−
	52	24	Unseg.	−
	53	434	18–20	+
25-somite *cont.*		433	21–24	−?
		432	Unseg.	+
	54	398	Unseg.	−
	55	163	Oto.	+
		164	1– 4	−
	56	517	1– 5	+
	57	956	19–22	+
		954	23–25	+
	58	1,009	19–21	−?
	59	1,016	18–21	−
		1,017	22–25	+
	60	1,117	22–25	−
		1,118	22–25	−
	61	1,175	4– 8	−
		1,174	13–16	−
	62	1,187	1– 3	−
		1,189	4– 7	+
		1,190	8–10	−
	63	1,238	5– 8	+
		1,239	Unseg.	+
	64	1,317	1– 4	−
		1,316	5– 7	−
	65	1,409	5– 8	−
		1,410	9–11	−
	66	1,424	1– 4	+
		1,425	12–15	+
26-somite	67	53	6–10	−
	68	92	10–12	−?
		91	21–24	+?
	69	248	1– 5	−?
		247	22–26	+
	70	893	24–26	+
		894	24–26	+
		895	Unseg.	−
		896	Unseg.	+
	71	884	24–25	−
		883	Unseg.	−
	72	1,011	14–17	+
	73	1,087	7–10	+
	74	1,103	1– 4	−
		1,104	5– 7	+
		1,101	8–11	−
		1,102	12–15	−
	75	1,199	6– 8	−
		1,200	9–12	−
	76	1,125	1– 4	−
	77	1,229	Oto.	+
		1,228	5– 8	−
		1,227	9–12	+
	78	1,271	1– 3	−
		1,272	8–11	+
	79	1,308	1– 5	+
		1,309	6– 9	+
		1,307	10–12	+

were established here by the 45-somite stage or slightly earlier. Apparently melanoblasts did not begin to enter the epidermis lateral to the somites and anterior to the wing buds (level of somites 1–13) or between wing buds and leg buds (level of somites 19–25) until the 45-somite stage, and they did not appear to be established in these regions until somewhat later. This was difficult to ascertain for the region anterior to the wing buds because of difficulties encountered in getting grafts from this region to survive, which accounts for the meager data recorded for this region.

grafts were obtained from gill arches 3–5, but, as Figure 1 shows, these lie lateral to somites 1–6; and, since transplants of the epidermis lying between these somites and the gill arches contained no melanoblasts until the 46- or 47-somite stages (Fig. 3), it seems unlikely that they acquire a supply of the migrating melanoblasts prior to this time.

C. INTO EPIDERMIS OF LIMB BUDS

1. *Along the proximodistal axis.*— Series C was designed to determine the time of arrival of melanoblasts at different points along the proximodistal axis and along the dorsoventral axis of the limb buds. In order to follow the migration of the melanoblasts from the base toward the tip of the limb bud, only the dorsal epidermis was transplanted. Owing to difficulties of handling and successfully transplanting extremely small strips of epidermis, no attempt was made to divide the isolated dorsal epidermis of very young limb buds (from donors younger than 43 somites) into proximodistal pieces. Instead, this entire piece of epidermis was regarded as roughly equivalent to that covering the basal third of the limb bud of embryos 43 somites and older. Beyond the 43-somite stage the dorsal epidermis isolated from the limb buds was divided into proximal,

TABLE 2

MIGRATION OF MELANOBLASTS INTO EPIDERMIS OF GILL ARCHES

Regions of Gill Arches Tested	Somite Age									
	34	35	36	37	38	39	40	41	42	43
Junction of maxilla and mandible	+	+	+		−		+			+
Body of the maxilla						−	+		+	
Body of the mandible						−			−	
Hyoid arch		+				−	−			+
		−								

Since the melanoblasts arrived lateral to the mesencephalon very early in development and soon thereafter lateral to the rhombencephalon, one might expect the adjacent gill arches to receive their melanoblast supply earlier than more posterior regions. Data on these regions are limited, since the grafts from these areas did not seem to survive well. Although 52 transplants of gill-arch epidermis were made, only 16 grafts could be recovered. The results of these transplants are given in Table 2; even though very sketchy, they suggest that the gill-arch ectoderm, except for the region at the junction of maxilla and mandible, does not become supplied with melanoblasts much before the epidermis at the bases of the limb buds. No successful

middle, and distal pieces and each piece was transplanted separately. Data on the migration of melanoblasts into the basal third of the limb bud were also presented in the preceding section. Only 45 identifiable grafts of epidermis from the middle and distal thirds of the limb buds were recovered. Admittedly, this is too small a series to offer much information concerning the exact time at which the middle and distal thirds of the limb buds become invaded by prospective pigment cells. However, since there is available at present no other information in the literature concerning these matters, the results of these transplants have been summarized in Table 3. Inspection of this table shows that, whereas the dorsal epidermis of the basal third of the wing bud yielded no negative grafts after the 44-somite stage and the dorsal epidermis of the basal third of the leg bud no negative grafts after the 45-somite stage, the middle and distal thirds of both continued to yield negative grafts in older stages. This is particularly true for the distal third and indicates that the entire ectodermal covering of the limb bud does not receive its melanoblast supply simultaneously but that there is a progressive migration of melanoblasts from the base toward the tip.

TABLE 3

MIGRATION OF MELANOBLASTS INTO EPIDERMIS OF LIMB BUDS

Range of Incubation Hours	Somite Stage	Wing Buds								Leg Buds							
		Basal Third				Middle Third		Distal Third		Basal Third				Middle Third		Distal Third	
		Dorsal		Ventral		Dorsal		Dorsal		Dorsal		Ventral		Dorsal		Dorsal	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
87	36	1															
87	39										1						
83–93	40	1*	1								5						
84½–96	41		4							1	1						
79½–98	42	2 2*	4								4						
83¼–98¼	43	2	7							3	1				1		1
84¾–100¾	44	9	1		5			1		4	6			1	2		1
85¾–103	45	4			2				1	10	3		1*	3	3		3
86¾–111½	46	4		1						10			2	1	1		
88¾–99¾	47	1		2	2					2			1	1			
88¼–113	48			1	2					5		1	2	3	2	1	1
93¼–116	49								3	4		2	3				3
94–111½	50									1				1	1		1
91–96	52									1*				1			
105	Undet.															2	
110	Undet.					2											
113	Undet.					1		1									
117	Undet.								2								

* Classification of graft uncertain.

2. *Along the dorsoventral axis.*—To determine whether there is any difference in the time of arrival of melanoblasts in the epidermis on dorsal and ventral surfaces of the limb buds, epidermis was also isolated from the ventral surface of

the basal third of both limb buds and tested for the presence or absence of melanoblasts (see Table 3). Although the dorsal epidermis of the wing bud consistently contained melanoblasts after the 44-somite stage, melanoblasts had not reached the ventral epidermis until the 46-somite stage, and this region did not yield consistently positive tests for their presence, even at the 48-somite stage. Very similar results were obtained in the leg bud. Here the dorsal epidermis consistently contained melanoblasts after the 45-somite stage, yet the first positive tests for their presence in ventral epidermis were obtained in the 48-somite stage, and a high proportion of negative tests continued to occur even in 49-somite embryos.

Again the evidence indicates that the entire epidermis of the limb bud does not receive its supply of melanoblasts at one time but that there is a progressive dorsoventral migration of these cells, in addition to a progressive proximodistal migration.

II. MIGRATION OF MELANOBLASTS INTO STRUCTURES OTHER THAN EPIDERMIS AND ITS DERIVATIVES

DuShane (1944) states that in adult birds melanophores are usually restricted quite sharply to the skin and its derivatives; nevertheless, he presents scattered experimental data which suggest a considerably wider distribution of melanoblasts during embryonic stages. Consequently, DuShane suggests that perhaps the paucity or absence of nonepidermal melanophores in most adult birds is due not to a selective migration of melanoblasts to the epidermis alone but rather to an inhibition of the differentiation of those melanoblasts which do reach other regions or to a degeneration of any melanophores that appear elsewhere than in the skin.

A. DISTRIBUTION OF MELANOPHORES IN NONEPIDERMAL STRUCTURES OF BARRED PLYMOUTH ROCK EMBRYOS

In order to discover whether melanoblasts do differentiate into melanophores elsewhere than in the skin and, if so, where and when, the following observations were made. Normal Barred Plymouth Rock embryos ranging in age from $6\frac{1}{2}$ to 21 days of incubation were examined under a dissecting microscope for the distribution of visible nonepidermal melanophores. In each age group at least three embryos were usually studied. It should be emphasized that these observations do not trace the progressive migration of melanoblasts but rather record the regions where melanoblasts which presumably arrived earlier are undergoing differentiation into melanophores and are consequently becoming visible.

$7\frac{1}{2}$–8 days.—Melanophores first became visible within the epidermis of the dorsal surface; the viscera and coelomic linings remained free from them until approximately 8 days. In one 8-day embryo a few appeared along the mesonephric ducts and the adjacent blood vessels, particularly along the posterior portions of the latter.

$8\frac{1}{2}$–9 days.—The internal melanophores were slightly more prominent than before. The greatest concentration seemed to be along the renal ducts and blood vessels. In some cases they extended from the vessels into the coelomic lining covering the mesonephroi. A few were also seen along the epaxial muscles and in the region of the ribs.

$9\frac{1}{2}$–10 days.—There was a slight increase in general internal pigmentation. The heaviest concentration of melanophores was found in the posterior coelomic lining close to the umbilicus. They spread out from here along the urogenital ducts toward the mesonephroi and meta-

nephroi. In two embryos the pigment cells were concentrated rather heavily in the ventral ligament of the gizzard, giving it a decidedly slate-colored appearance. A few scattered melanophores were found in the coelomic lining over the ribs.

10½–11 days.—Pigment cells were still most numerous in the posterior coelomic lining, extending from here into the ventral ligament of the gizzard and along the sex ducts, ureters, and the blood vessels between the excretory organs. There seemed to be an indication of a preferential accumulation near blood vessels, particularly the large dorsal vessels. Melanophores also showed a tendency to cluster in the coelomic lining and mesenteries in the region of the developing gonads, but they did not seem to spread out into the intestinal mesentery for any great distance.

11½–12 days.—More melanophores were visible in the ventral ligament of the gizzard than had been previously; some were even present in the falciform ligament. Their concentration was heaviest along blood vessels and nerves. All specimens had a scattering of melanophores along the main dorsal blood vessels and in the coelomic lining covering the mesonephroi, metanephroi, and the ribs. The urogenital ducts and vessels were outlined by the branched, black melanophores.

12½–13 days.—As before, the posterior portion of the coelomic lining contained many pigment cells, and there was a noticeable concentration of them within the ventral mesentery of the gizzard and the falciform ligament. There was the usual anterior concentration along the mesonephric ducts in the region of the kidneys. The melanophores were clustered around the blood vessels leading into the intestinal mesentery, radiating from a center of concentration between the mesonephroi and the gonads, so that the large blood vessels of this region were bordered with pigment cells.

13½–14 days.—A fine sprinkling of melanophores was present throughout the coelomic lining. They were also located in the deeper tissues surrounding the ribs. The posterior coelomic lining and ventral ligament of the gizzard were well pigmented, and there was the usual scattering of melanophores along the urogenital ducts. There seemed to be a tendency for the melanophores to congregate at the root of the intestinal mesentery just anterior to the gonads and to spread out from here both into the mesentery and laterally into the peritoneum over the metanephroi. For the first time, pigment cells appeared in the gonads of the two male embryos of this group. In one the mesial surface of the right testis was pigmented, except for the most posterior quarter; in the second the entire inner surface of the left testis was lightly pigmented, and the right testis bore a sprinkling of melanophores along its inner border. The third embryo, a female, showed no trace of gonadal pigmentation.

14½–15 days.—The distribution of internal melanophores remained unchanged, except that a number of them were now visible in the mesorectum and in the region of the rectal caecae.

15½–16 days.—The heaviest concentration of melanophores was found in the posterior coelomic lining, the ventral mesentery of the gizzard, and the ligament extending from the right posterior coelomic wall to the liver and gall bladder. There seemed to be fewer melanophores along the blood vessels and ducts leading to the excretory organs, but the number increased along the anterior border of the mesonephroi, and they were seen spreading laterally in the coelomic lining over the metanephroi. Of the three

male embryos in this group, only one showed pigment cells in the gonads. In this embryo the mid-third of the left gonad was black with pigment.

16½–17 days.—Internal melanophores were distributed as before. In one embryo they followed the large blood vessels out into the intestinal mesentery as far as the spleen and pancreas. They radiated out from the region of the dorsal aorta lying between the gonads.

17½–18 days.—There was little change in the distribution of the internal melanophores. Two male embryos had pigmented gonads; in one the mid-third of the right testis contained melanophores; in the other the anterior half of the right testis and the mid-third of the left were a deep black.

18½–19 days.—These older embryos were unchanged except for a more conspicuous extension of the melanophores along the vessels of the intestinal mesentery toward the spleen. The male embryo in this group had pigmented areas along the inner surfaces of both testes.

19½–20 days.—Melanophore distribution was unchanged. One male had a right testis which was solid black except for the anterior and posterior tips.

20½–21 days.—Only one embryo was examined; it seemed to have fewer melanophores in the mesenteries and coelomic lining than did those previously examined. In striking contrast to this general decrease in the visceral melanophores was the heavy pigmentation of the testes; the left gonad was almost solid black, while the middle, inner third of the right testis was deeply pigmented.

Such observations on normal Barred Plymouth Rock embryos are sufficient to demonstrate that there is no differential migration of melanoblasts to the skin alone, that they must migrate extensively into many other parts of the body, and that in many other parts of the body of the embryo they are capable of differentiating into melanophores. Their absence in many corresponding regions of the adult must mean that such melanophores later degenerate. This degeneration apparently does not begin until about the time of hatching; only the one specimen of this age suggested a considerable reduction in the number of internal melanophores compared with earlier stages.

B. TIME OF MIGRATION OF MELANOBLASTS INTO SOME MESODERMAL DERIVATIVES

Since the observations recorded above demonstrate conclusively that melanoblast migration occurs in directions other than toward the skin, an attempt was made to determine when melanoblasts actually enter certain nonepidermal structures. These experiments were not intended to be at all extensive but were designed merely to give an indication concerning the time of arrival of melanoblasts within a few representative organs of mesodermal origin. Since the testis frequently contained a heavy concentration of melanophores, an attempt was first made to determine when melanoblasts enter the gonad. Small segments of gonads were excised from Barred Plymouth Rock donors which had been incubated 116–67 hours and were transplanted to the base of the wing bud of White Leghorn host embryos. Although twenty-five hosts survived, the graft could be identified in only eleven of these. These grafts, which had been contributed by four donors, were uniformly negative.

A second series of operations, which entailed transplanting small segments of the mesonephroi and accompanying mesonephric ducts from Barred Plymouth Rock donors to White Leghorn host embryos, gave markedly different results. The data for this experiment are as follows: at 118 hours, 3 negative and

2 positive grafts; at 121 hours, 1 negative and 2 positive grafts; thereafter only positive grafts (3 at 125 hours, 2 at 130 hours, and 1 at 135 hours). The ten positive grafts occurred on hosts which possessed large patches of pigmented down feathers on the wing and breast. The fact that the negative grafts were obtained only from the younger donors (118–21 hours) and that donors older than 125 hours yielded only positive grafts strongly suggests that melanoblasts are migrating into this organ about this time.

Each Barred Rock donor embryo younger than 20 somites contributed two grafts; one graft consisted of that section of the blastoderm which included Hensen's node (labeled *1* on Fig. 4, *A*); the other (labeled *2*, Fig. 4, *A*) included only the primitive streak region. In donors older than 20 somites the neural folds

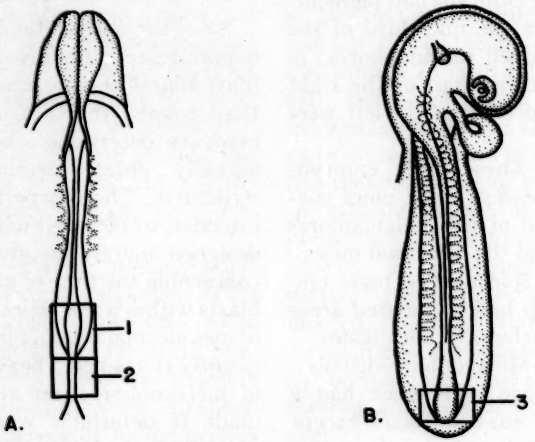


FIG. 4.—Diagrams of Barred Rock donors, showing regions grafted. *A* represents a stage with incompletely fused neural folds and shows the location of Hensen's node (1) and primitive streak grafts (2). B represents an embryo with a definitive tail bud (3).

III. CAPACITY OF HENSEN'S NODE AND THE TAIL BUD TO PRODUCE MELANOBLASTS

Holmdahl (1925*a*, *b*) maintained that all structures of the chick embryo posterior to the 27th to 28th somites (epidermis, neural tube, notochord, somites, gut, etc.) originate from the tail bud. If this is true, a question arises concerning the source of the melanoblasts which give rise to the melanophores responsible for the pigmentation of these posterior parts of the body. Does the tail bud possess the capacity to give rise to melanoblasts, or must they migrate into those parts of the body derived from the tail bud from more anterior regions? In order to answer this question, experiments were conducted as follows.

have closed, and Hensen's node has enlarged and has become part of the developing tail bud, the primitive streak being no longer visible. Subsequent to this stage the entire tail-bud region posterior to the end of the neural tube was excised and transplanted (graft labeled *3*, Fig. 4, *B*); each older donor therefore contributed a single graft. Such grafts were implanted into the coelom of White Leghorn embryos as described by Hamburger (1942). Nine grafts, consisting of primitive streak alone obtained from donors ranging in age from 14 to 21 somites,

were recovered; none of these had produced pigment cells.

The melanophore-producing capacity of grafts of Hensen's node from the earlier embryos and of tail buds from older embryos is indicated in Tables 4 and 5. It will be seen that Hensen's node in the young embryos produced melanophores in ten out of fifteen hosts in which the graft could be identified; the age of the donor embryo did not seem to be the deciding factor as to whether a graft was positive or negative. The tail buds, however, seemed more variable in their capacity to produce pigment cells. Tail buds from 21- to 27-somite donors produced pigment cells in only thirteen out of thirty-three operations in which the graft could be recovered, i.e., in about 39 per cent of the cases. By contrast, the tail buds from 28- to 46-somite donors produced pigment cells in forty-one out of fifty operations in which the grafts could be recovered, i.e., in about 82 per cent of the cases. Thus the stage of the donor was an important factor in the capacity of the tail bud to produce melanophores. In making isolations of the tail bud as illustrated in Figure 4, *B*, the possibility always existed that the posterior end of the neural tube might be included in the isolate and that grafts would be positive for pigment cells only when this happened. In order to check on this point, a number of coelomic grafts of tail buds, some positive for pigment cells and some negative, were fixed in Bouin's fluid, sectioned, stained with haematoxylin and eosin, and studied histologically. The results are recorded in Table 6.

Although in many cases the grafts seemed to lack organization, histogenesis and even organogenesis were quite good. Cartilage, feather germs, epidermis, smooth muscle, ganglia, nerves, and pigment cells were easily recognized. In certain grafts (such as N, G, B, and Q_1) the gut was also well formed, showing secre-

TABLE 4

MELANOPHORE-PRODUCING CAPACITY OF HENSEN'S NODE IN BARRED ROCK EMBRYOS

Somite Age (No. of Somites)	Positive	Negative
5		1
12	1	1
13	1	
14	1	
15	1	
17	1	2
18	1	1
19	3	
20	1	
Total	10	5
Per cent	66	33

TABLE 5

MELANOPHORE-PRODUCING CAPACITY OF THE TAIL BUD IN BARRED ROCK EMBRYOS

Somite Age (No. of Somites)	Positive	Negative
21	2	5
22	1	2
23	1	4
24	3	1
25	2	4
26	1	1
27	3	3
28	1	
30	4	
31	5	1
32	3	
33	3	
34	4	
35	3	2
36	2	
38	2	
39		1
40	5	2
41	1	
42	1	1
43	5	
44	1	1
46	1	1
Total	54	29
Per cent	65	35

tory mucosa and smooth-muscle layers arranged in the longitudinal ridges characteristic of the digestive tract of gallinaceous birds. In the rest of the grafts the identification of gut was more difficult; in many of these an epithelium of a columnar mucus-secreting type unlike the epidermis was present on the surface of the graft and was underlain by layers of smooth muscle. This epithelium seemed to be gut epithelium, in which case the graft must have turned inside out, reversing the normal arrangement of the tissue layers. Such grafts are recorded as questionable for the presence of gut.

TABLE 6

TISSUES AND ORGANS DEVELOPING IN TAIL-BUD GRAFTS

	Embryo																			
	D	B	S	L	M	H	Q	Q_1	R	A	E	P	T	N	I	K	F	C	G	O
	Somite Stage																			
	18	21	21	21	22	22	23	24	25	26	40	19	19	22	25	27	30	31	32	40
Epidermis	+	+	?	+	+	+	+	...	+	+	+	+	+	+	+	+	+	+	+	+
Feather germs	+	+	...	+	+	+	+	...	...	+	+	+	...	...	+	+	+	+	+	+
Cartilage	+	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+
Gut	...	+	?	...	?	?	?	+	?	...	...	?	?	+	?	?	...	?	+	...
Smooth muscle	...	+	+	...	+	+	+	+	+	...	...	+	+	+	+	+	...	+	+	...
Skeletal muscle	+	+	...	...	+	...	+	...	...	...	+	...	...	...	?	?	...	...	...	...
Ganglion cells	...	...	...	...	...	+	...	+	...	...	...	+	...	+	...	...	...	...	+	+
Nerves	...	+	+	?	+	+	+	+	+	...	?	+	...	+	...	+	...	...	+	+
Neural tube	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+
Pigment cells	...	...	...	...	...	...	...	...	...	...	...	+	+	+	+	?	+	+	+	+
Blood vessels	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Examination of Table 6 reveals that there is no correlation between the presence or absence of pigment cells and the presence or absence of ganglion cells or nerves. The neural tube appeared only in one graft (O) as a short tube surrounded by cartilage, extending only through five or six sections. It contained a central canal, and both ependymal and mantle layers were plainly visible.

The possibility also existed that melanoblasts might have migrated posteriorly along the dorsal surface of the neural tube and into the tail bud posterior to the neural tube. If such were true, in some cases these melanoblasts might be isolated with the tail bud, or in other cases the tail bud might not contain them when isolated. The extent of the posterior migration of the melanoblasts, therefore, might be the deciding factor in determining whether a graft would, or would not, produce melanophores. This possibility seems unlikely, however, in view of the fact that melanoblasts were almost never found lateral to the neural tube over the segmental plates (see Fig. 2), indicating that migration of melanoblasts was not under way at all at the posterior end of the embryo at the time that the tail buds were isolated.

Thus it appears that Hensen's node possesses the ability to give rise to melanophores and that the definitive tail bud possesses this ability to a marked extent but that there exists a transition

period between recognizable node and definitive tail bud, during which the capacity of this region to form pigment cells is unpredictable.

Discussion

I. Anteroposterior gradient in early melanoblast migration

The close correlation between the presence of melanophores in transplants and the development and migration of the neural crest as figured by Holmdahl (1928) verifies the work of Ris (1941) and substantiates the hypothesis that pigment cells in birds originate from the neural crest. Holmdahl has clearly demonstrated that in young avian embryos the neural crest does not migrate out from the dorsal midline until after a closed neural tube has formed, except in the region of the mesencephalon, where crest development is precocious. Anterior to the 27th or 28th somite level, the the neural tube forms by the fusion of the neural folds, a process which does not begin until after 6 somites have formed and which is not completed until about the 20-somite stage. Thus somite formation spreads caudad much more rapidly than does the onset of neural-crest migration; and one could predict that during early stages of development there would be a considerable lag between the formation of a given somite and the arrival of melanoblasts above it. This prediction agrees perfectly with the data on stages prior to 26 somites (Fig. 2). But during later developmental stages (period of secondary body development of Holmdahl) the lag between the establishment of a closed neural tube and the formation of the most posterior pair of somites disappears. Out of a continuous mass of cells there emerges dorsally a solid strand, which, as rapidly as it forms, becomes canalized to form a neural tube (Holmdahl, 1925*a*, *b*; Schumacher, 1927); out of this same mass of cells, epidermis, notochord, gut epithelium, etc., arise simultaneously. For our discussion it is unimportant whether these posterior regions differentiate from totipotent cells of the tail bud, as postulated by Holmdahl and Schumacher, or whether they are formed by rapid division of the cells lying between the previously differentiated structures and the tail bud, as maintained by Graper (1926, 1927). Whatever the manner of their formation, the important fact remains that now somites and neural tube are increased simultaneously. Assuming that the neural crest also arises in the posterior trunk and caudal regions at the same time as does the closed neural tube, we could predict that in older embryos there would be a close correlation between the formation of the most posterior pair of somites and the arrival of melanoblasts above it. This prediction also agrees perfectly with the data on stages after the formation of the 30th pair of somites. Between the 26- and 30-somite stages lies a period of transition, during which migration often lags slightly behind somite formation.

The results of testing for the presence or absence of melanoblasts above the somites are very consistent until the 19- or 20-somite stage, in that they are almost always present above the first few somites and nowhere else. Likewise, they are very consistent after the 26-somite stage, in that melanoblasts are present over almost all the somites (Fig. 2). But between the stages of 19- and 27-somites it is impossible to predict whether melanoblasts will be present above given somite levels; a random mixture of positive and negative grafts is obtained during these stages (Fig. 2). Indeed, this, too, might be anticipated in view of the behavior of the neural crest. Careful ex-

amination of Holmdahl's illustrations (1928) reveals markedly different ratios between the rate of neural-crest formation and the rate of new somite formation at different stages of embryonic development, the most rapid development of the crest occurring between 20- and 23-somite stages subsequent to the complete fusion of the neural folds. Prior to this period the slow posterior extension of the crest means that melanoblasts are well out over relatively few of the most anterior somites. Consequently, epidermis isolated from these regions will consistently contain melanophores. Immediately after the 20-somite stage, melanoblasts are migrating laterad above the somites over a considerable portion of the anteroposterior axis almost simultaneously. Slight individual variations in the rate of neural-fold fusion or in the rate of crest formation or migration become critical at this time; the epidermis of slightly precocious embryos will contain melanoblasts at most levels of the body; that of slightly retarded embryos will yield negative grafts over large areas of the embryonic axis (compare embryos 42 and 46 with embryo 40, Table 1). Not only will individual developmental variations become critical at this time, but slight alterations of operative technique will play a major role in determining whether crest cells are, or are not, included in a given transplant. Such operative variations are unavoidable and probably account for the apparent breaks in the anteroposterior gradient in onset of migration, which was noted in certain embryos tested between the 20- and 26-somite stages.

A puzzling characteristic of the onset of migration of melanoblasts involves the level of the most anterior somites. Epidermis from this level fairly consistently yields melanophores in 16- to 18-somite stages (Fig. 2), but thereafter yields a considerable number of negative grafts even as late as the 36-somite stage. By contrast, the epidermis over other somite levels, once it has become consistently positive, remains so in all later stages. Actually, one might suspect that the neural crest above the anterior somites would produce few melanophores. Niu (1947) found that explants of posterior cranial crest in *Triturus* had a low melanophore content; and Hörstadius and Sellman (1946), Landacre (1921), Stone (1929), and Raven (1931) have demonstrated that in amphibians the crest of this region makes significant contributions to the branchial skeleton. Although precise data pertinent to this matter are lacking for birds, Yntema (1944) mentions that removal of the posterior cranial crest in the chick results at times in deficiencies of the hyoid arch; and Holmdahl (1928) suggested that the crest in the regions of the mesencephalon and the anterior and middle rhombencephalon supplies the mesenchyme for the gill arches. Moreover, the posterior cranial crest of the chick also contributes to the formation of the glossopharyngeal and vagus ganglia (Yntema, 1944; Jones, 1942) and to the cervical sympathetic trunks and ganglia (Yntema and Hammond, 1945). In view of this evidence, it is tentatively suggested that the melanophores which differentiated in grafts removed from 16- to 18-somite embryos might have been migrating crest cells carried over with the epidermal transplants. These cells, if left in the donor, would have differentiated into nervous or gill-arch derivatives but, being only labilely determined at this time, had their course of development switched by transplantation to a foreign environment. In grafts isolated slightly later, no melanophores would appear either because the crest cells were now rigidly determined to form nonpigment cells or because they had moved away from the

area without entering the epidermis. Niu (1947) has demonstrated the labile character of neural-crest derivatives during the early developmental stages of *Triturus*. Explanation of the crest during the neurula stage revealed that, while prospective pigment cells were the major component of the trunk crest, they constituted only a very small percentage of the derivatives of the posterior cranial crest; the latter gave rise mostly to "giant" cells, believed to be precartilage cells. But, if he explanted the posterior cranial crest into coelomic fluid, it would give rise to a higher percentage of pigment cells than would identical cultures grown in salt solution. He concluded that at this time the cells of the posterior cranial crest must be only labilely determined as precartilage and that, when the environment was changed, some could differentiate in a direction other than toward their prospective fate, viz., into melanophores.

II. MEDIOLATERAL MIGRATION OF MELANOBLASTS

Heretofore, *Triturus* has been the only animal in which the pathways of melanoblast migration have been completely charted. In this genus, cells leaving the trunk neural crest take one of three paths: (1) Most of the cells move directly laterad, the spread continuing unless, as in *T. torosus*, an obstacle such as the yolk border prevents further dispersal. There is, therefore, a distinct anteroposterior gradient along the trunk in both lateral migration and subsequent pigmentation (Twitty, 1936, 1944; Twitty and Bodenstein, 1944). (2) Cells originating from the anterior trunk crest swing anteriorly to populate the head (Niu, 1947). (3) Cells in the region of the posterior trunk crest swerve caudally to enter the tail bud (Twitty, 1944).

Since some evidence had been presented that melanoblasts enter the wing bud before they penetrate the leg bud (Watterson, 1938, 1942; Willier and Rawles, 1940), it was assumed that, like *Triturus*, the chick would show a clear anteroposterior gradient in the mediolateral migration of the melanoblasts. Figure 3 shows that this expectation was not realized. Not only do melanoblasts penetrate the epidermis of both limbs at almost the same somite stage, but they apparently reach these regions before they penetrate into lateral trunk epidermis anterior to the wing or between the limb buds. Further, the data for the gill arches, though scanty, seem to indicate that melanoblasts do not reach even these most anterior levels of the body much before they extend into the basal segments of the appendages. These data all indicate that in the developing avian embryo the pattern of lateral migration is complicated by the presence of the developing limb buds, which are lacking in the young amphibian larva. In the chick some stimulus from the limb buds either increases the rate of migration of melanoblasts at these levels or causes melanoblasts migrating laterad in other levels of the trunk to converge toward the limb buds, leaving areas anterior to the wing and between the limb buds free of melanoblasts during the early migratory period. The precocious penetration of melanoblasts into the epidermis of the leg bud in the 44-somite stage (84–100 hours of incubation) is rather surprising. Watterson (1938) found that leg-bud mesenchyme of 92- to 94-hour donors produced negative, or only weakly positive, grafts; and Willier and Rawles (1940) presented data suggesting that melanoblasts enter the epidermis of the leg bud between 103 and 132 hours of incubation. It seems possible that the discrepancy between the data of the present study and those formerly published may be caused partly

by slightly different conditions of incubation, which become apparent when incubation age is used as the criterion of development, and partly by removal of epidermis or mesenchyme from different areas along the limb axis. As shown in the present study, only the dorsal epidermis of the basal third of the leg yields consistently positive grafts at the 45-somite stage, that of more distal or ventral areas continues to test negatively in far later stages.

There are several lines of evidence in the present study which suggest that the Barred Rock embryo resembles *T. torosus* in respect to a cranial migration of cells from the anterior trunk crest. First, as Table 1 shows, between the 20- and the 26-somite stage there were only two embryos (Nos. 31 and 23) in which the epidermis covering the most anterior somites tested positively and the epidermis lying directly posterior tested negatively. Second, this hypothesis would explain the tardy appearance of melanoblasts in the epidermis covering the gill arches or lying lateral to the more anterior somites. Third, it would explain the reappearance and late establishment of melanoblasts in the epidermis covering the first somites, a region from which they disappeared at the 19-somite stage. Since grafts of epidermis from the most anterior cranial regions did not show the fluctuations characteristic of the occipital area, it seems likely that only the posterior portions of the head are invaded by the trunk melanoblasts.

In the chick there seems little evidence that cells of the posterior trunk crest furnish melanophores for the tail bud or its derivatives. Both Hensen's node and the tail bud into which it becomes incorporated can give rise to pigment cells when transplanted. Moreover, comparison of Table 5 and Figure 2 shows that in many cases positive grafts were obtained from tail buds removed before neural-crest migration was under way at the levels of the more posterior somites. This fact argues convincingly against any posterior migration of melanoblasts into the tail buds prior to their transplantation.

III. CAUSAL FACTORS IN DETERMINING PATHWAYS OF MELANOBLAST MIGRATION

Although intended primarily to be descriptive rather than analytical in nature, the present study enables us to examine the current theories of melanoblast migration in the light of actual migratory patterns established in a living embryo. Roughly speaking, the factors influencing melanoblast migration can be grouped in two classes: (*A*) facilitating factors, which aid in the dispersal of the cells, and (*B*) blocking factors, which prevent or hinder migration.

A. FACILITATING FACTORS

Three suggestions as to possible facilitating factors are to be found in the literature. Of these, that made by Rosin (1943) of a chemical or tissue affinity between melanoblasts and certain types of epidermis seems to have little application in the Barred Rock chick, since melanoblasts have been demonstrated in many nonepidermal structures.

On the basis of data obtained from explantation studies (Twitty and Bodenstein, 1939, 1944; Twitty, 1944, 1945) Twitty has suggested that the prime factor responsible for melanoblast dispersal is a mutual repulsion mediated by substances diffusing from neighboring melanoblasts. This hypothesis is adequate to explain the early mediolateral migration of melanoblasts in the chick and the migration of cells from the anterior trunk crest into the head if, as previously suggested, posterior cranial-crest cells do not differentiate into melanophores. How-

ever, it does not explain the precocious migration of melanoblasts into the limb buds or the dorsoventral migration within the limb buds.

Since it is a well-known fact that neural-crest derivatives tend to migrate and congregate along the surfaces of formed structures, thigmotaxis may play an important role in influencing the migration of the melanoblasts. During the early development of the crest, histological preparations clearly show the cells migrating between the inner surface of the epidermis and the upper border of the somites or between somites and neural tube. In older embryos the clusters of melanophores found along blood vessels, nerves, tendons, and ducts have been mentioned by Ris (1941), Hamilton (1940), and Humm (1942). The present study of the viscera and coelomic cavity of the normal Barred Rock embryo further emphasizes this fact. But the tendency of melanoblasts to migrate along formed structures does not explain their attraction to the limb buds. At the time that melanoblasts penetrate the limbs, histological examination reveals neither an orientation of the compact mesenchyme nor any invading nerve fibers along which migration might occur. A few blood vessels are present, but, since they are equidistant from dorsal and ventral epidermis, it is difficult to see how migration from these foci could give rise to the dorsoventral gradient which seems to be present.

Perhaps, as Weiss (1929, 1934, 1945) has demonstrated for Schwann cells, fibroblasts, and nerve fibers, melanoblasts can also travel along ultramicroscopic paths formed by an orientation of the micellae of the substrate. Since he has shown in tissue cultures that dehydration of the culture medium by rapidly growing cells can bring about such an orientation of the substrate, it does not seem too rash to suggest that the rapidly dividing cells of the limb buds may bring about a similar orientation of the micellae of the tissue fluid to form paths for the migrating melanoblasts. Differential growth in various regions of the body or within an organ might thereby play an important role in determining the patterns of pigment-cell migration.

B. BLOCKING FACTORS

While there is some evidence to suggest the presence of certain blocks to pigment-cell migration in the Barred Rock embryo, speculation as to their nature at the present time lies in the realm of pure theory. Since melanophores are present to some extent in certain of the mesenteries, one is inclined to accept only with some reservation the suggestion of Ris (1941) that a structural block prevents the migration of melanoblasts between body wall and mesenteries. If such a block is present, it must be incomplete at the points of penetration of nerves and blood vessels, for in many instances melanophores accompany these structures out into the mesenteries.

Since the transplantation experiments seem to indicate that melanoblasts are entering the mesonephroi by 123 hours of incubation and since pigment cells are not present in gonadal grafts as late as 167 hours of incubation, one wonders whether some physical or physiological block prevents melanoblast migration into the developing gonads at this stage of development. One also wonders whether the permanent presence of such a block in the female might not also explain the curious fact that the ovary of the Barred Rock is rarely pigmented, while the testis frequently contains large masses of melanophores.

There is a certain amount of evidence to suggest a third definite block to mel-

anoblast migration in the developing Barred Rock embryo. Chicks of this breed have dark, nonbarred down plumage except on the ventral surface, the wing tips, and the region of the dorsal head spot, which lies above the posterior rhombencephalon. In these isolated areas there are present varying numbers of white or grayish feathers. Watterson (1942), impressed with the lack of pigmentation in the apical wing feathers in spite of an abundance of melanophores in the underlying mesodermal parts of the wing, suggested that, unless melanoblasts enter the epidermis before a certain stage in the differentiation of the latter, they are prevented from entering in some way until down-feather development has been completed. The apical wing feathers would, therefore, be nonpigmented, since melanoblasts would arrive in this region too late to enter the epidermis. This explanation might account equally well for the presence of the head spot and for the lack of pigmentation of the ventral surface, since evidence previously presented suggests that melanoblasts become established in the epidermis of these areas relatively late in development.

Summary

1. Certain epidermal and nonepidermal regions of Barred Rock embryos were tested for their ability to produce melanophores when transplanted to the wing bud or coelom of White Leghorn embryos. By this method a time schedule was obtained for the departure of melanoblasts from all levels of the neural tube and for their arrival in various regions of the developing embryo.

2. Melanoblasts first migrate beneath or into the epidermis lateral to the neural tube in the region of the mesencephalon of the 8-somite embryo. By the 19-somite stage migration is under way as far posterior as the level of the first few somites. Between the 20- and the 27-somite stage lateral migration occurs over all the somites save the last five. After the 30-somite stage migration keeps pace with the formation of new somites but rarely occurs in the area of the segmental plates. The onset of migration of melanoblasts follows approximately an anteroposterior sequence.

3. The time at which the melanoblasts become established in the epidermis covering the rhombencephalon at the level of the first few somites is highly variable. It is suggested that this fact may account for the variable size and shape of the white or grayish head spot which is characteristic of the Barred Rock chick.

4. Melanoblasts first spread laterad to the somites at the levels of the limb buds, becoming established in the base of the wing bud in the 44-somite stage and in the base of the leg bud in the 45-somite stage. Prospective pigment cells do not, however, become established in the lateral epidermis anterior to the wing bud or between the limb buds until somewhat later in development. The gill arches are also relatively slow in receiving their melanoblast supply. Melanoblasts seem to be directed precociously toward the limb buds. Some of the possible factors involved are discussed.

5. Regional grafts within the limb buds indicate that the entire epidermis of the limb does not receive its melanoblast supply at one time but that there are both proximodistal and dorsoventral gradients of melanoblast migration within the limbs. Delay in the arrival of melanoblasts at the wing tip is probably correlated with the formation of white down plumage here.

6. Examination of the coelomic cavity

and viscera has revealed that melanophores differentiate within many nonepidermal structures during embryonic life. This indicates that there is no selective migration of melanoblasts into the epidermis. Their absence in these structures in adult birds must mean that they later degenerate.

7. Transplantation of segments of mesonephroi and gonads from Barred Rock donor embryos to White Leghorn host embryos demonstrates that melanoblasts apparently enter the mesonephroi at about 125 hours of incubation, but their presence cannot be demonstrated in the indifferent gonads by 167 hours of incubation. The testes frequently contain an extensive network of melanophores after the 14th day, but the ovaries are unpigmented.

8. Both primitive streak and Hensen's node of Barred Rock donors of less than 20 hours of incubation and the tail-bud region of older Barred Rock embryos were tested for their ability to produce melanophores when transplanted to the coelom of White Leghorn host embryos. The primitive streak lacks this capacity, but both Hensen's node and the definitive tail bud possess this ability to a high degree. The young, developing tail bud is unpredictable in this respect. Histological examination of nodal or tail-bud grafts has not revealed any correlation between the presence of nervous tissue and the presence of melanophores.

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THE EFFECT OF SEITZ FILTRATION ON THE PROTEIN CONTENT AND PRECIPITIN REACTION OF DILUTED ANTIGEN SOLUTIONS[1]

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INTRODUCTION

MANY investigators use Seitz filtration to maintain sterility in blood sera and other antigens employed in serological studies. The Sietz E.K. metal filter which employs thick disposable asbestos filter pads is quick and easy to use, may be thoroughly cleaned, and is inexpensive. According to Larkum (1929), less than 1 per cent contamination follows routine Seitz filtration as compared with 4 per cent contamination encountered when a Mandler filter is regularly used.

A survey of the literature revealed that Browne, in 1942, reported changes in hydrogen-ion concentration attendant upon the Seitz filtration of various aqueous solutions. He found that distilled

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water became increasingly alkaline when Seitz-filtered. Ten per cent sugar solutions exhibited a corresponding shift to alkalinity when filtered, but acetate and phosphate buffers showed no such change. A similar study in 1944 by Webb, Irish, and Lyday confirmed these results and further noted that a 5 per cent solution of human blood serum underwent little alteration in pH when filtered. These authors attributed the increased pH reading of some solutions after filtration in part to the presence of alkaline substances derived from the filter pad. Seiler (1932) and earlier workers had noted a release of magnesium ions from the pads. A chemical analysis (Webb, Irish, and Lyday) of filter disks from two different manufacturers showed magnesium to be present in both.

The specific effect of finely divided asbestos fiber was demonstrated by the inactivation of various dilute solutions of hormones, drugs, and antisera brought in contact with it, as shown by Coplans (1913–14). According to Wyard (1913–14), amboceptor and complement were removed from diluted immune sera in the presence of crude and washed asbestos fiber from several geographic localities.

Chopra and Roy (1936, 1938) investigated the effect of Seitz filtration on the hemolytic property of snake venoms. They found that when a 0.01 per cent solution of venom was filtered, it was no longer hemolytic but that filtration had little effect on a 0.1 per cent venom solution. Finally, these authors demonstrated the removal of specific bacterial hemolysins from antisera which had been subjected to Seitz filtration. They attributed these results to action of the asbestos fiber of the filter pad in either eliminating the active principle by a process of adsorption or altering it in some way.

Preliminary work in this laboratory indicated that a much-filtered antigen solution gave a lower precipitin titer in comparison with the same solution freshly made and unfiltered, when tested with a single antiserum under identical conditions. Since any effect that a Seitz filter may have on the substance passing through it should be taken into account when using such filtered solutions in serological tests, a detailed study of the nature and extent of these filtration changes in various antigenic preparations—blood sera of various mammals and hemoglobin solutions—was made, and the results of this study are reported in this paper.

MATERIALS AND METHODS

Dilutions of beef, human, orangutan, cat, dog, and rat blood sera were used to test the effect of Seitz filtration on antigen solutions. Two highly purified hemoglobin preparations of mammalian origin were also filtered and tested. These antigens had been stored undiluted in a sterile condition under refrigeration, and all were free of bacterial contamination at the time of using. Fresh dilutions of these antigens were prepared in either 1.8 per cent saline or phosphate-buffered 0.9 per cent saline solution and were filtered immediately.

Seitz filters of two different sizes were used, and filtration of the solution was achieved with suction or pressure. The large filter required a size 6 pad, the small filter used a size 3 pad, and the effective filtering diameters were 50 and 30 mm., respectively. The pads were all of approximately uniform thickness, about 3 mm. each. The changes caused by filtration were measured in the following three ways:

1. The protein content of the solutions before and after filter passage was calculated by using the sensitive colorimetric micromethod for the determin-

tion of nitrogen described by Johnson (1941). Water blanks, standard nitrogen solutions, and duplicate assays were run, and the grams protein per milliliter of the test solutions were calculated. Then, in order to derive relative protein values in reference to concentrated 100 per cent solutions of blood serum, the protein values obtained were multiplied by the dilution factor in question. The latter values were then converted to grams protein per 100 ml. of pure solution.

All nitrogen present was assumed to be protein nitrogen, and any error in the protein calculations attributable to small amounts of nonprotein nitrogen was not taken into consideration. That such an error was present, but negligible, was shown in some of the later tests, where precipitation by trichloracetic acid was used as a protein indicator.

2. The reactivity of the antigens before and after filtering was measured by means of the precipitin test. The interfacial titers were obtained against chicken antisera of known potency. These antisera had been "aged" in the refrigerator until all exhibited a maximum stable titer. Chicken antisera were used because of their availability and their uniformly high titers. Doubling dilutions of the test antigens were made in small serological tubes with 1.8 per cent saline solution, and 0.1 ml. of antiserum was carefully layered beneath 0.5 ml. of each antigen dilution. The dilution of the last tube with a defined ring at the interface after incubation for 1 hour was taken as the end-point (titer) of this test. All tests were run in duplicate, and, as the final readings fell within the range of error accepted for this type of test, the readings were averaged.

3. Quantitative measurements of the precipitate volume were made with antisera produced in rabbits in standardized Van Allen thrombocytocrits according to the method of Boyden and Baier (1929). Preliminary tests of each new antiserum used were made against various antigen dilutions to determine a dilution which would fall on the right-hand side (antibody excess) of the reaction curve, that is, beyond the region of maximum precipitation. Tests were run in duplicate, and measurements were averaged.

Each experimental procedure employing beef serum was repeated on a second serum dilution, prepared, filtered, and tested at a later date. Similar results were always derived with these, indicating the uniformity and constancy of the filtration changes which occurred. Representative data from the experiments undertaken are presented in this paper.

RESULTS

I. COMPARISON OF FILTRATION EFFECTS ON DILUTED ANTIGENS PASSED THROUGH LARGE FILTERS

Table 1 records the relative protein content, calculated from total nitrogen assays, and the precipitin reaction resulting when 0.5, 1.0, and 2.0 per cent solutions of beef serum were filtered through large Seitz filter pads of a 50-mm. effective filtering diameter. Successive aliquots of 10 ml. were removed as filtration progressed, and all tests were performed on each aliquot.

In general, the first 10 ml. to pass the filter of any of the concentrations tried showed a markedly reduced protein content and corresponding decreases in the ring-test titer and volume of precipitate. The first 10-ml. aliquots of the 0.5 and 1.0 per cent solution each gave a negative ring-test titer and no measurable precipitate when mixed with a homologous antiserum. There were measurable amounts of nitrogen, but it was probably nonprotein nitrogen. The first filtered aliquot of the 2.0 per cent solution gave a greatly reduced interfacial titer but

no measurable antigen-antibody precipitate; the nitrogen content was greater than with the higher dilutions of the antigen; and, since a precipitin reaction occurred, part of this nitrogen must have been protein nitrogen.

The second 10 ml. of the 0.5 per cent antigen solution that filtered through still did not give a precipitin reaction and showed nitrogen quantities similar to the first 10-ml. portion. On the other hand, a definite precipitin reaction could be demonstrated by the two methods employed when the second 10 ml. of the filtered 1.0 and 2.0 per cent solutions were tested, and there was a corresponding increase in the protein content of these filtered aliquots when compared to the first 10-ml. collections.

The results yielded by the later aliquots indicate that, when a 0.5 per cent solution is being filtered, the protein is being removed continuously, though in lesser amounts, through the total 80 ml. tested; with the 1.0 per cent solution, removal continued through about the first 50 ml.; and, with the 2.0 per cent solution, practically all the protein that was removed disappeared in the first 20 ml.

In addition to the beef serum dilutions, 2.0 per cent solutions of human, orangutan, cat, dog, and rat sera were filtered and the protein concentrations determined. The results were all very comparable. Figure 1 shows diagrammatically the results secured with dog and cat antigens. Two diluted solutions, made from crystalline preparations of beef and dog hemoglobin, were also filtered. The former solution of beef hemoglobin contained the higher percentage of protein. The results secured with this relatively pure protein duplicated the data secured with the serum proteins.

TABLE 1

COMPARISON OF RELATIVE PROTEIN CONTENT, INTERFACIAL TITERS, AND PRECIPITATE VOLUMES BEFORE AND AFTER FILTRATION OF 0.5, 1.0, AND 2.0 PER CENT BEEF SERUM SOLUTIONS THROUGH LARGE SEITZ FILTERS
(50-Mm. Effective Filtering Diameter)

Aliquots	Gm. Protein per 100 Ml.			Interfacial Titers			Vol. of Precipitate (Cc.)		
	0.5 Per Cent Solution	1.0 Per Cent Solution	2.0 Per Cent Solution	0.5 Per Cent Solution	1.0 Per Cent Solution	2.0 Per Cent Solution	0.5 Per Cent Solution	1.0 Per Cent Solution	2.0 Per Cent Solution
Before filtration....	7.00	7.00	7.25	153,600	102,400	102,400	0.0140	0.0070	0.0090
1st 10 ml. filtered..	0.25*	0.33*	0.72	0†	0†	4,800	None	None	None
2d 10 ml. filtered...	0.16*	0.77	2.90	0†	4,800	25,600	None	0.0030	0.0050
3d 10 ml. filtered...	0.25*	3.44	6.00	400	76,800	51,200	0.0014	0.0031	0.0051
4th 10 ml. filtered..	1.28	4.44	6.12	9,600	76,800	102,400	0.0040	0.0030	0.0067
5th 10 ml. filtered..	3.25	5.25	7.12	19,200	102,400	102,400	0.0037	0.0033	0.0090
6th 10 ml. filtered..	4.41	5.44	.....	51,200	102,400	.....	0.0039	0.0040	.....
7th 10 ml. filtered..	5.00	5.69	.....	102,400	102,400	.....	0.0080	0.0042	.....
8th 10 ml. filtered..	5.37	5.88	.....	102,400	102,400	.....	0.0110	0.0041	.....

* Probably nonprotein. † Lowest dilution tried was 1:200.

II. FILTRATION EFFECTS OF SMALL FILTERS

It is to be expected that filtration through smaller pads would cause less alteration of the antigens in the filtering process. The data recorded in Table 2 show the effects of filtration on two different 2.0 per cent beef serum solutions,

designated as "D_1" and "D_2", through pads with an effective filtration diameter of 30 mm. The type of data recorded is similar to that given in Table 1, but 5-ml. aliquots instead of 10-ml. aliquots were removed for testing.

The first 5 ml. removed showed a decided reduction in protein content, a great loss in the volume of precipitate, but only a slight, though significant, reduction in the interfacial titer. These results show the limitations of the latter method in studies of this kind. The second 5 ml. removed and tested showed a much higher protein content and a large increase in precipitate volume when compared to the first 5 ml., though both were lower than the unfiltered antigen dilution. The interfacial test method did not distinguish this filtered sample from the unfiltered solution. Beginning with the third aliquot, no great change was perceptible by either type of immune test, but the protein content was slightly less than the original material.

III. EFFECT OF REPEATED FILTRATION

In serological studies, especially those involving relationship studies, it is often necessary to refilter a solution if an infection appears in it. The purpose of this experiment was to ascertain the effects of repeated filtration of a 2.0 per cent beef serum solution through new large pads. Seven aliquots of 50 ml. each were prepared and filtered, the number of filter passages varying consecutively from one to seven. After each aliquot had been filtered the desired number of times, the three types of tests previously described were performed to detect any filtration changes. The final volume of solution was measured after each filtration series, and it was found that there was a total

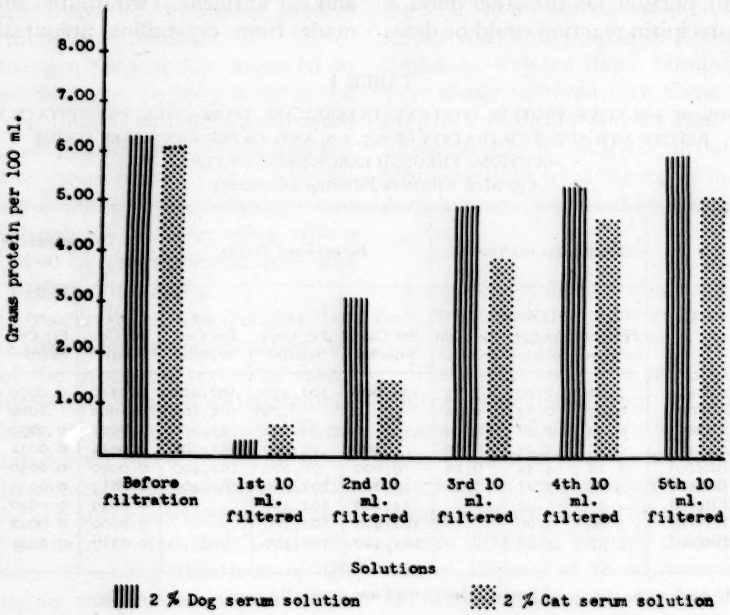

FIG. 1.—Effect of filtering 2 per cent solutions of carnivore blood sera through large Seitz filters (50 mm. diameter).

18-ml. loss after seven passages through new pads.

The results of these experiments are recorded in Table 3. A demonstrable loss of protein and a definite reduction in precipitate volume were noticeable after one filtration, but the interfacial titrations showed no significant decrease. A second filtration resulted in another substantial loss of protein and a further reduced precipitate volume, and now there was a significant decrease in interfacial titers recorded. Three filtrations completely eliminated the serological reactions, but there were detectable amounts of nitrogen. However, all solutions filtered three or more times failed to give a positive protein reaction when tested with a 10 per cent trichloracetic acid solution.

Another experiment was performed in the same manner, using small filter pads, and only protein determinations were made. The results confirmed the previous data, but it was necessary to carry through seven filtrations before all the protein nitrogen was removed.

DISCUSSION

The results of all experiments reported in this paper are in close agreement as

TABLE 2

RELATIVE PROTEIN CONTENT, INTERFACIAL TITERS, AND PRECIPITATE VOLUMES AS AFFECTED BY FILTRATION OF 2.0 PER CENT BEEF SERUM SOLUTIONS THROUGH SMALL SEITZ FILTERS (30-Mm. Diameter)

Aliquots	Gm. Protein per 100 Ml.		Interfacial Titers		Precipitate Volume (Cc.)	
	D_1	D_2	D_1	D_2	D_1	D_2
Before filtration	7.00	7.06	51,200	102,400	0.0131	0.0129
1st 5 ml. filtered	2.00	2.25	6,400	25,600	0.0049	0.0048
2d 5 ml. filtered	5.13	6.00	38,400	76,800	0.0118	0.0104
3d 5 ml. filtered	6.00	6.25	25,600	76,800	0.0130	0.0120
4th 5 ml. filtered	6.25	6.75	38,400	102,400	0.0129	0.0120
5th 5 ml. filtered	6.50	6.75	51,200	102,400	0.0131	0.0119

TABLE 3

RELATIVE PROTEIN CONTENT, INTERFACIAL TITERS, AND PRECIPITATE VOLUMES OF CONSTANT AMOUNTS (50 ML.) OF 2.0 PER CENT BEEF SERUM SOLUTION BEFORE AND AFTER FILTRATION THROUGH LARGE SEITZ FILTERS (50-Mm. Diameter)

No. Times Filtered	Gm. Protein per 100 Ml.	Interfacial Titers	Precipitate Volume (Cc.)	Final Aliquot Volume (Cc.)
Before filtration	7.00	102,400	0.0107	50
1	4.19	51,200	0.0056	46
2	1.06	12,800	0.0020	42
3	0.75	0*	None	40
4	0.34	0*	None	34
5	0.23	0*	None	31
6	0.25	0*	None	30
7	0.22	0*	None	32

* Lowest dilution tried was 1:100.

regards the potent activity of the Seitz filter pad in protein removal when dilute blood serum antigens are filtered. In no case with the solutions tested was this filtration effect absent.

The results obtained with the two sizes of filter pads showed major differences in filtration effects to be related to filter size. At least three times as many small filter passages were required to obtain a comparable decrease in protein values. Thus the relative activity of the two filters can be correlated with the effective surface area of their pads, which is 1,964 sq. mm. for the large pad and 707 sq. mm. for the small. However, dilute antigens filtered more than once lost so much protein in filter passage, regardless of filter size, that it is recommended that refiltration be avoided.

The protein loss induced by Seitz filtration may introduce a large error into precipitin tests and should always be taken into consideration when diluted antigens are filtered. Since the loss of protein is greatest in the first portions to pass the filter pad, as shown in the experimental results, the difficulty of achieving and maintaining sterility in small quantities of rare or hard-to-obtain blood sera is obvious. Only very large volumes of a 2.0 per cent solution should be filtered at one time, and the entire filtrate should be pooled or the early collections discarded to minimize the loss of protein. The experimental data indicate that with more dilute solutions even this would prove an inadequate safeguard. An important protein loss was found to be extended through a larger number of aliquots, hence affecting a greater volume of filtrate, when 0.5 per cent solutions were tested in comparison to 1.0 per cent solutions or when both these serum concentrations were compared with our standard 2.0 per cent preparation. Evidently, after a certain amount of protein material had been filtered, the filter pad became saturated, and very little additional protein was removed, as shown by the higher protein values of the last filtered aliquots in each experiment. This was also substantiated by the fact that drying a used filter pad which had exercised its maximum action in protein removal did not restore its capacity for additional removal. Therefore, it would seem best to avoid filtering anything less than a 2.0 per cent solution of serum antigen through large filters.

The data presented throughout this paper illustrate the effect of reduced protein content in the filtrates on interfacial titers and quantitative determinations of precipitate volume. The relatively greater sensitivity of the latter tests made them useful for detecting small changes not obvious from the interfacial end-points. All quantitative measurements for a given solution were made with a single antiserum and can therefore be compared. Comparisons between different solutions are not valid because different antisera may have been used in order to run the large number of tests required.

The effect of some possible modifying factors, beyond filter size, was studied in connection with observed changes in protein content. Since the two surfaces of the Seitz filter pads are different in appearance, it seemed of interest to test the influence, if any, of the position of the filter pad. One surface can be distinguished by the imprint of fine screening, and the other surface appears rough and fibrous. Reversal of the filter pad within the filter did not alter the character and extent of the changes observed in two similar solutions upon filtering. Neither did the method of filtration influence the results in these experiments. Suction filtration seemed to be as efficient in pro-

tein removal as filtration by pressure. Various other factors, such as the appearance of fine cracks in the filter pad which sometimes accompany pressure filtering, and the use of either a buffered 0.9 per cent saline solution or unbuffered 1.8 per cent solution did not appear to modify the changes caused by filtration.

The results recorded from tests on other blood sera showed that filtration changes were not limited to any peculiarity associated with beef serum. The preliminary data secured with dilute hemoglobin solutions correlated nicely with results obtained with serum antigens. Since this phase of the work was not extensive, it will be necessary to repeat these studies on hemoglobin; and experiments are being planned that will use other relatively "pure" antigens, such as egg albumin, bovine albumin, and fractions of human plasma. The effect of filtering antisera of varying potency from the laboratory animals commonly used for antibody production should likewise be determined.

In view of the considerable and constant changes in diluted serum proteins following Seitz filtration, the importance of recognizing and allowing for the error thus introduced into serological tests cannot be overemphasized. Seitz filtration is a convenient and satisfactory method of maintaining sterility in blood sera only so long as its effects are known. The changes induced in dilute antigen solutions by filtration are particularly important in relationship studies, when small quantities of rare sera may be frequently filtered. In all quantitative work, serum dilutions based on actual protein content should be prepared according to the calculated protein values from nitrogen assays performed after, and not before, the material is subjected to final filtration.

SUMMARY

1. The changes induced by Seitz filter pads in dilute antigenic solutions were analyzed with regard to the effect of the following variables; antigen concentration, antigen diluent, filter size, number of filter passages, order of filtration, position and pretreatment of filter pad, and methods of filtration.

2. The first and second 10-ml. aliquots of 2.0 per cent beef serum solutions to pass through a large Seitz filter pad underwent a marked loss of protein. The reduced protein content, based on total nitrogen assay, was correlated with diminished interfacial titers and reduced volumes in the thrombocytocrit measurements of precipitate.

3. Filtration of solutions more dilute than 2 per cent, namely, 1.0 and 0.5 per cent beef serum preparations, showed a marked protein loss to be extended to greater volumes of the filtrate. The greater the initial dilution, the more complete the protein removal in a 50-ml. volume.

4. A small Seitz filter removed proportionally less protein from 2.0 per cent beef serum solutions than did large Seitz filters.

5. Repeated filtration through large-size fresh filter pads resulted in the complete disappearance of protein in a 50-ml. volume of 2.0 per cent beef serum filtered only three times. Protein was completely removed from a like amount of 2.0 per cent solution passed through seven small filter pads in succession.

6. Blood serum dilutions from several other animal species showed changes similar to those undergone by beef serum solutions of equal concentration subsequent to Seitz filtration.

7. The importance of these filtration effects on diluted antigens and the error which is thereby introduced into serological studies were discussed.

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GROWTH OF PROTOZOA IN GLASSWARE CLEANED WITH SULPHURIC ACID–POTASSIUM BICHROMATE SOLUTION

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IN VIEW of the results obtained by Laug (1934) and by Richards (1936), many biologists have been reluctant to use sulphuric acid–bichromate solution for the cleaning of glassware to be used in growth experiments. Laug found that ten water rinses were not sufficient to remove all the bichromate from glassware treated with the usual bichromate cleaning solution, and Richards showed that small amounts of bichromate, such as might be present after ten rinses, were toxic to various organisms. Richards found, for example, that the addition of 0.1–1.0 γ potassium bichromate per milliliter of culture medium caused a 22–47 per cent reduction in growth of *Saccharomyces cerevisiae* within 24 hours after inoculation. These same concentrations of bichromate also caused injury and death to *Oscillatoria limosa*, two species of *Spirogyra*, and the developing eggs of *Amblystoma punctatum*. Similarly, Henry and Smith (1946) reported that bichromate concentrations of 1–10 γ/ml were very toxic to growth of *Staphylococcus aureus*. They also found, at least in some cases, that a small amount of bichromate could be detected after ten rinses.

In the author's early studies of the nutritional requirements of flagellates, the glassware was washed with soap, thoroughly rinsed, and steamed. When later results showed that *Astasia* sp., one of the colorless flagellates, was capable of continuous growth in a purely inorganic medium, it was considered advisable to clean all glassware with sulphuric acid–potassium bichromate solution as an added precaution against organic compounds being present (Schoenborn, 1946). The possibility remained, however, that the use of glassware cleaned with this solution was in part responsible for the slow growth and low yields observed in the inorganic medium. This possibility has been investigated. Since no reports are available on the effect of small

amounts of bichromate on growth of any Protozoa in pure culture, two additional species were studied: a green flagellate, *Euglena gracilis*, and a ciliate, *Tetrahymena geleii*, strain E. The results were somewhat different from what might have been expected on the basis of previous reports and are therefore described below.

MATERIALS AND METHODS

The culture vessels (125-ml. Pyrex Florence flasks) were filled with the cleaning solution, which was allowed to remain in the vessels 24 hours, and the flasks were then rinsed 2, 4, 20, or 100 times with cold water. When vessels were given 20 rinses, the 10th rinse water was allowed to remain in the vessels overnight, while the 20th, 40th, 60th, and 80th rinses were allowed to remain overnight in flasks rinsed 100 times. Those vessels receiving 100 rinses should contain no bichromate which could diffuse out into the culture medium (Laug, 1934), and thus served as control vessels. Additional control vessels were prepared by boiling new flasks in Calgonite[1] solution for 1 hour followed by a total of 20 rinses. The 10th rinse was allowed to remain in these flasks overnight, after which they were boiled in tap water for 1 hour and then given the final 10 rinses. In all cases the last 2 rinses were made with distilled water from a Pyrex glass still. Glassware used in preparation of media was cleaned with the bichromate solution followed by 100 rinses. The cleaning solution employed was usually prepared by adding potassium bichromate crystals directly to concentrated sulphuric acid (sp. gr. 1.84) to obtain a saturated solution, although in some cases about 300 ml. of a saturated aqueous solution of bichromate were added to 9 lb. of acid.

[1] Manufactured by Calgon, Inc., Pittsburgh, Pa.; contains about 60 per cent alkaline silicates and 40 per cent sodium metaphosphate.

Glassware cleaned as outlined above was used for growth studies of all three protozoan species in an organic medium (medium A) consisting of 1 per cent Difco Bacto-tryptone and 0.2 per cent KH_2PO_4 in distilled water. Growth of the two species of flagellates was also studied in inorganic media. The medium used for *Euglena gracilis* (medium B) was: NH_4NO_3, 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; KH_2PO_4, 0.2 gm.; $CaCl_2 \cdot 2H_2O$, 0.1 gm.; $FeCl_3 \cdot 6H_2O$, 0.0025 gm.; $MnCl_2 \cdot 4H_2O$, 0.0001 gm.; double distilled water, 1.0 liter. Medium C, used for autotrophic growth of *Astasia* sp., was identical with the purely inorganic medium used previously (Schoenborn, 1946). After being adjusted to a suitable pH with HCl or NaOH, the media were measured into culture vessels and autoclaved at 120° C. for 15 minutes.

Flasks of media so prepared were inoculated from a pure culture in the same medium. *Euglena* cultures were grown at a north or shaded east window, cultures of the other two species in darkness, and all cultures at room temperature. Initial pH was 6.30–7.00 in different experiments. In those series with inorganic media the initial pH was usually 0.02–0.03 pH units lower in twice-rinsed acid-bichromate cleaned flasks than in other flasks of the same series, but differences greater than 0.01 pH units were not noted in experiments with organic medium. The density of population was determined after inoculation and at various times during the growth period (Schoenborn, 1946, 1947), and growth curves plotted from the results obtained. It is not necessary to reproduce these curves, since the results can be expressed by noting the generation time during the logarithmic phase of growth and the maximal population levels. Statistical significance

was determined by the analysis of variances and/or Student's *t* test.

EXPERIMENTAL RESULTS

ORGANIC MEDIUM

In these experiments the flasks contained 50 ml. of medium, and the initial population density was less than 500 organisms per milliliter in all but Series VI. Since the inocula (1 ml. per flask) were taken from cultures in the logarithmic phase of growth, no lag phase was observed in the experimental cultures (Phelps, 1935; Schoenborn, 1947).

Four experiments were carried out with *Astasia* sp. In Series I and II no significant differences were observed either in generation time or in maximal population level in bichromate-cleaned flasks rinsed 4 times, and those rinsed 20 times or cleaned with Calgonite (averages shown in Tables 1 and 2). Growth in flasks rinsed only twice following bichromate cleaning was not significantly

TABLE 1

GENERATION TIME, IN HOURS, DURING LOGARITHMIC PHASE OF GROWTH

Series	Species	Medium	Glassware Cleaned with				
			Calgonite	Bichromate Solution Followed by			
				2 Rinses	4 Rinses	20 Rinses	100 Rinses
I–II	*Astasia* sp.	Organic	7.64	.....	7.58	7.49	.....
III	*Astasia* sp.	Organic	.....	7.36	.....	7.44	.....
IV	*E. gracilis*	Organic	30.38	30.31	.....	30.61	30.12
V	*T. geleii*	Organic	14.84	14.58	.....	14.65	.....
VI	*T. geleii*	Organic	5.33	5.38	.....	.....	5.34
VII	*E. gracilis*	Inorganic	108.9	99.1	.....	.....	99.6
XI–XIII	*Astasia* sp.	Inorganic	33.72	41.50	.....	33.89	33.65
XIV	*Astasia* sp.	Organic	.....	8.08	.....	.....	8.19
XV	*Astasia* sp.	Inorganic	.....	37.45	.....	.....	37.44

TABLE 2

LARGEST POPULATIONS OBSERVED DURING MAXIMUM STATIONARY PHASE OF GROWTH

(In Organisms per Milliliter)

Series	Species	Medium	Glassware Cleaned with				
			Calgonite	Bichromate Solution Followed by			
				2 Rinses	4 Rinses	20 Rinses	100 Rinses
I–II	*Astasia* sp.	Organic	287,167	.....	300,967	292,934	.....
III	*Astasia* sp.	Organic	.....	340,200	.....	331,733	.....
IV	*E. gracilis*	Organic	324,600	359,800	.....	378,300	375,500
V	*T. geleii*	Organic	11,320	11,880	.....	12,440	.....
VI	*T. geleii*	Organic	104,353	108,400	.....	.....	96,267
VII–X	*E. gracilis*	Inorganic	1,594	1,976	.....	.....	1,890
XI–XIII	*Astasia* sp.	Inorganic	2,470	2,408	.....	2,479	2,329
XIV	*Astasia* sp.	Organic	.....	420,000	.....	.....	447,333
XV	*Astasia* sp.	Inorganic	.....	3,652	.....	.....	3,804

different from that in flasks rinsed 20 times (Ser. III) or 100 times (Ser. XIV). *Euglena gracilis* was studied in Series IV, and, while the generation times were similar in all vessels, the maximal populations were significantly lower (at the 5 per cent level) in the Calgonite-cleaned flasks. A significant difference in yield was not observed, however, in a check series using Calgonite-cleaned flasks and vessels rinsed 20 times following bichromate cleaning. *Tetrahymena geleii* was used in Series V and VI. Because of the relatively poor growth observed in Series V, thiamine hydrochloride (1 γ/ml) was added to the medium for Series VI. Growth was better in this latter series, and in both series the generation times and yields were not significantly different in the various flasks.

INORGANIC MEDIA

Better growth of *Astasia* sp. in inorganic medium has been routinely observed in flasks containing relatively small volumes of solution as compared to larger volumes. For this reason only 30–40 ml. of inorganic media per flask were used for both *Astasia* and *Euglena*. Cultures used to inoculate the experimental series were 19–46 days old, and in some cases a lag phase was observed in the newly inoculated cultures. Inocula of 1–2 ml. per flask resulted in initial population densities ranging from 38 to 172 organisms per milliliter in different series.

The growth of *E. gracilis* was studied in Series VII. Generation times were similar in the bichromate-cleaned flasks, whether rinsed 2 or 100 times, but appeared to be somewhat slower in the vessels cleaned with Calgonite (Table 1). This difference, however, did not prove to be significant. The yield was significantly lower (at the 5 per cent level) in the Calgonite-cleaned vessels, and three additional experiments (Ser. VIII–X) were therefore carried out to study the maximum population levels. No significant difference was observed in these three series between the yields in the various vessels; nor was the difference in yield significant when the four experiments were considered together. Results shown in Table 2 are averages of the maximum populations observed in Series VII–X.

Three series, XI–XIII, were carried out with *Astasia* sp. in inorganic medium. Bichromate-cleaned vessels rinsed 2 and 100 times were used as before, plus similarly cleaned flasks rinsed 20 times, this latter procedure having been followed by the author in previous studies of *Astasia* sp. where cleaning solution was used. In each series the generation times were similar in all flasks except those treated with cleaning solution followed by 2 rinses. Here significantly slower growth was observed. However, the maximal populations were not significantly different in the various flasks. Averages of these three experiments are shown in Tables 1 and 2.

CUMULATIVE EFFECTS

The foregoing results led to the question of whether continual growth in organic medium in twice-rinsed bichromate-cleaned flasks would eventually cause inhibition of growth. *Astasia* sp. was used to investigate this possibility in Series XIV. Richards (1936) has pointed out that greater bichromate concentrations might be expected when small volumes of medium are placed in glassware treated with cleaning solution followed by insufficient rinsing. This might be expecially true when the medium is autoclaved in the vessels as in these experiments. Therefore, in Series XIV only 20 ml. of medium A were used per flask, the flasks being bichromate-cleaned and rinsed either 2 or 100 times.

Eleven serial transfers were concurrently carried out in each of the two types of flasks, the first transfers receiving inocula from the same stock culture. Each transfer was allowed to grow 4 days and was then used to seed the next transfer, 1-drop inocula being employed. Thus, at the end of 10 transfers, one line had been growing for a 40-day period in flasks rinsed 100 times, the other line in flasks rinsed only twice. No determinations of population densities were made during these first 10 transfers, but in the 11th transfer counts were made and growth curves plotted. As noted in Tables 1 and 2, the generation times were similar in all flasks, as were the maximal populations. The observed differences did not prove to be significant.

Series XV, similar to the above experiment, was run to determine whether cumulative effects occurred during growth of *Astasia* sp. in inorganic medium C. Because of slower growth in this medium, it was necessary to incubate each transfer for longer periods (20–32 days), and to use 1-ml. inocula when seeding the next transfer. Seven serial transfers were carried out, covering a period of about 6 months, with population densities being determined in both the 6th and the 7th transfers. In both these transfers the generation times and yields were not significantly different in the twice-rinsed flasks and in those rinsed 100 times (averages shown in Tables 1 and 2).

DISCUSSION AND SUMMARY

Of the culture vessels used in this investigation, it can be assumed that those cleaned with Calgonite were bichromate-free, since only new flasks were used for this purpose. Glassware cleaned with the sulphuric acid–potassium bichromate solution and rinsed 100 times, as outlined above, should be free of any bichromate which could diffuse out into the culture medium; vessels rinsed 20 times should also be free of bichromate; but those flasks which received only 2 rinses undoubtedly contributed appreciable quantities of bichromate to the medium (Laug, 1934; Henry and Smith, 1946). Results of growth studies carried out in these different vessels permit the conclusion that the twice-rinsed bichromate-cleaned flasks do not inhibit growth of pure cultures of three protozoan species —*Astasia* sp., *E. gracilis*, and *T. geleii*— when grown in an organic medium. Nor was any cumulative effect, resulting in slower growth, observed when *Astasia* sp. was cultured in the organic medium in twice-rinsed flasks for a number of serial transfers. It would appear, therefore, that the usual bichromate cleaning solution can be used safely, at least for the protozoan species studied, in preparing glassware for experiments employing an organic medium such as that used in this investigation. However, the conclusion that small amounts of bichromate are not toxic to these Protozoa does not necessarily follow, since the bichromate concentration may have been reduced because of the binding action of the organic constituents of the medium (Henry and Smith, 1946).

When *E. gracilis* was cultured under autotrophic conditions, the twice-rinsed bichromate-cleaned flasks permitted growth comparable to that observed in flasks rinsed 100 times or to vessels cleaned with Calgonite. These results in purely inorganic medium permit the conclusion that small amounts of bichromate have no adverse effect on the growth of this green flagellate. Either *E. gracilis* behaves differently in this regard than do certain bacteria, yeast, algae, and developing salamander eggs (Richards, 1936;

Henry and Smith, 1946), or some difference in method is responsible for the difference in results.

The only evidence of bichromate toxicity in this study was observed when *Astasia* sp. was cultured in an inorganic medium in glassware rinsed twice after treatment with cleaning solution. Although the yield in these vessels was similar to that in better-rinsed bichromate-cleaned flasks or in flasks cleaned with Calgonite, the rate of growth was significantly slower in the twice-rinsed vessels. However, after *Astasia* sp. was cultured in serial transfers for several months in these twice-rinsed flasks, the growth rate was similar to that in flasks rinsed 100 times. This loss of bichromate toxicity during serial transfers may be due either to selection or to acclimatization. If the strain used to seed the first transfer contained organisms of different bichromate sensitivity, the resistant organisms may have been selected out during serial transfers so as to produce a strain whose rate of growth was not influenced by the bichromate concentration present in twice-rinsed flasks. Or the organism may have become acclimated to this concentration of bichromate. No attempt was made to obtain evidence bearing on these two possibilities.

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STUDIES IN THE PHARMACOLOGY OF THE HEART OF THE ORTHOPTERON, STENOPELMATUS

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ALTHOUGH numerous investigations have been made of the action of sympathomimetics, parasympathomimetics, and autonomic blocking agents on the hearts of many Crustacea and *Limulus*, the effects of drugs on the insect heart have, to the writer's knowledge, been studied in detail by few workers. Hamilton (1939) investigated the action of acetylcholine, atropine, and nicotine on the heart of the grasshopper, *Melanoplus differentialis* (Thomas). Yeager and his co-workers (1937, 1938) observed basic differences in the response to nicotine of the hearts of the adult roach, *Periplaneta americana* (Linn.), and the Southern Armyworm, the larva of the moth *Prodenia eridania* (Cram.). Prosser (1942) mentions in a footnote that in his laboratory acetylcholine has

been found to be acceleratory in the hearts of the roach (*Blatta orientalis*) and the honeybee. As early as 1877 Dogiel tested the effects of atropine, muscarine, and curare on heart rate in the larval mosquito, *Corethra* (Diptera: Culicidae), but his negative results are open to question, since he immersed the whole larva in drug solutions, which may never have penetrated to the heart.

The present work was undertaken to bring together in a study on a single species the knowledge of the action of the drugs used by the above workers, as well as of some other agents of physiological significance, and to compare the results obtained with the known action of the same drugs on the hearts of other arthropods, with the hope thereby of providing a basis of knowledge on which further experiments on the mechanism of heart control in insects may be conducted.

The large subterranean Orthopteron, *Stenopelmatus longispina* Brunner, known commonly on the Pacific Coast as the "Jerusalem Cricket" or "Potato Bug," has proved an excellent animal for these studies. The insects may be readily found under stones and grass clods, particularly in areas that have been under cultivation. They are large, easy to handle, and may be kept alive for weeks in small bottles containing moist soil; a piece of potato provides food and moisture. Insects must be kept in bottles singly, for under these conditions, at least, they are cannibalistic. In this work adult and late nymphal crickets were used.

MATERIAL AND METHODS

The abdomen of the insect was severed quickly from the thorax, and an incision was made separating the abdominal sternites from the tergites. The dorsum was then pinned down to a wax tray, and the strip of sternum, digestive tract, and gonad were carefully lifted away, exposing the delicate, thin-walled dorsal vessel. The preparation, thus isolated from any central nervous effects, was, except when under drug treatment, continuously bathed with fresh oxygenated saline, the wax tray to which the preparation was pinned being clamped at an angle so that the perfusion fluid flowed gently over the heart in a posteroanterior direction.

After experiments with the physiological salt solutions of Maloeuf (1935), Levy (1928), and Yeager and Hager (1934), none of which maintained a beat of long duration in *Stenopelmatus*, frog saline (NaCl, 6.7 gm.; KCl, 0.15 gm.; $CaCl_2$, 0.12 gm.; $NaHCO_3$, 0.15 gm. to a liter at pH 7.2) was used. In this solution controls were kept beating regularly and at a constant rate for as long as 4–5 hours. All tests were conducted at room temperature. The heart was observed through a binocular microscope, and the rate was recorded manually against time with a telegraph key and signal magnet on a smoked drum.

As had previously been observed by Yeager (*loc. cit.*) and others, heart preparations of this nature are not always constant in behavior, perhaps because of operational shock, nearness to molting, or some other unknown factor. Only those preparations were used which, after perfusion with saline for 5 or more minutes, exhibited a regular beat of constant rate. This constant rate differed in different preparations. As a "normal" might be taken a frequency of 41 beats per minute, an average for the rates of twenty-five of the preparations, the slowest of which maintained a constant rate of 30 beats per minute and the fastest, one of 52 beats per minute.

Drugs were administered directly to the heart from a pipette in such quantity that the saline surrounding the heart was immediately flushed away and replaced

by drug solution. Considerable difference could be observed in the threshold of response of different preparations to drugs, apparently depending on the condition or "sensitivity" of the pacemaker after isolation. That the variation in thresholds was not caused by a dilution of the drug or a delay in its reaching the heart was indicated by the immediate response of the heart to drugs above the threshold for the particular preparation. Similar variation has been observed by W. E. Garrey (1942) in his able investigations of the action of drugs on the ganglion of the heart of *Limulus*. Because of this variation, specific thresholds were not considered of significance except in cases where it was clearly demonstrated that they were markedly raised or lowered in a single preparation by previous treatment with another drug (Figs. 1, *A*, *B*, 2, *B*). In general, only those excitatory or inhibitory effects were considered significant or are illustrated which markedly raised or lowered the rate above or below the normal constant rate in saline (indicated by R' in the figures) for the particular preparation observed. Some seventy-five preparations were studied. No difference was discerned between the behavior of the hearts of adults and that of the hearts of larval instars in saline or under drug treatment.

Hamilton observed that his mechanocardiograms of the grasshopper were occasionally complicated by rhythmic contractions of the "alar" muscles when the preparation was being treated with drugs. No muscle bands are discernible in the extremely delicate diaphragm in *Stenopelmatus*, and no contractions other than those of the walls of the heart itself could be observed, even under treatment with high drug concentrations.

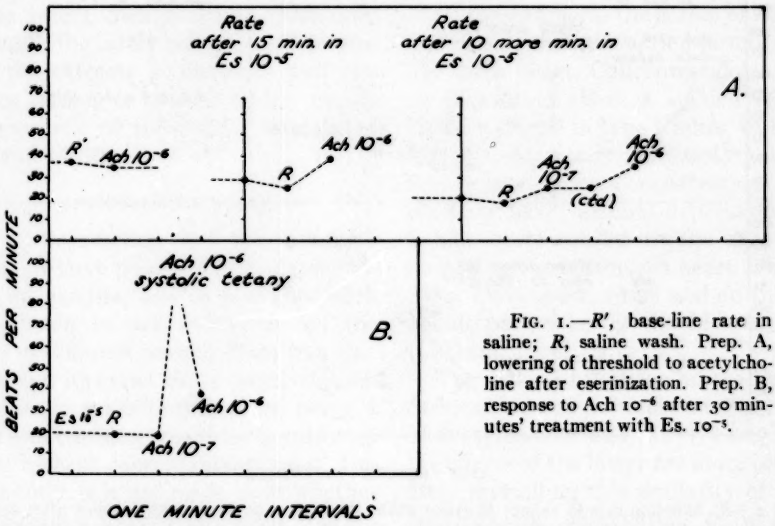

FIG. 1.—R', base-line rate in saline; R, saline wash. Prep. A, lowering of threshold to acetylcholine after eserinization. Prep. B, response to Ach 10^{-6} after 30 minutes' treatment with Es. 10^{-5}.

ACTION OF ACETYLCHOLINE

The acceleratory action of this drug on the heart of *Stenopelmatus* is similar in every way to its action demonstrated by many workers in Crustacea (Welsh, 1939*a*, *b*, 1940, 1942; Davenport, Loomis, and Opler, 1940; Davenport, 1941; Pros-

ser, 1942), on the ganglion of *Limulus* (Garrey, *loc. cit.*), the hearts of annelids (Prosser and Zimmerman, 1943) and the heart of the grasshopper, *Melanoplus* (Hamilton, *loc. cit.*).

In preparations which are sensitive but unpotentiated, low concentrations (10^{-6}) will accelerate the heart; and in such preparations concentrations as high as 10^{-4} will produce a great increase in rate, accompanied by a rise in tone and store normal beat apparently being dependent upon the state of depression of the pacemaker.

POTENTIATION BY ESERINE

To the writer's knowledge, although Means (1942) has demonstrated the presence of cholinesterase in the insect heart, potentiation of the action of acetylcholine by anticholinesterases has not been reported. The anticholin-

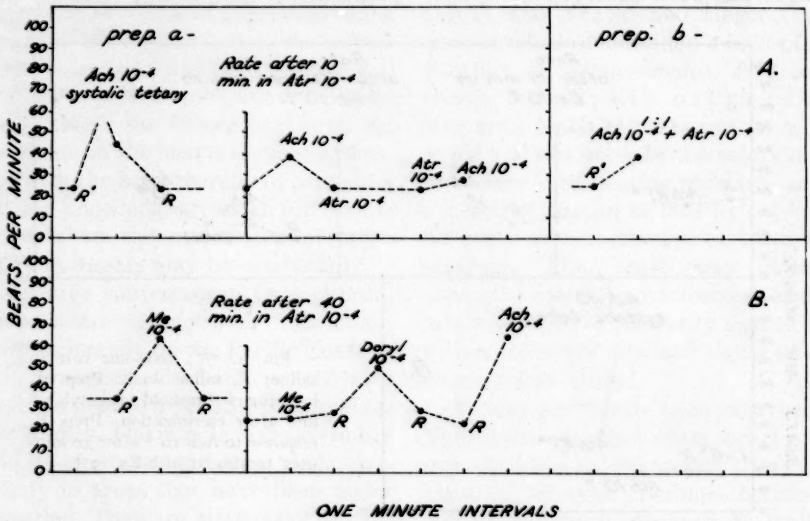

FIG. 2.—*R'*, base-line rate in saline; *R*, saline wash. Prep. *A*, *a*, action of acetylcholine after atropine. Prep. *A*, *b*, action of 1:1 acetylcholine and atropine. Prep. B, action of mecholyl, doryl, and acetylcholine after atropine.

transitory systolic tetany. This effect is perfectly reversible when the drug is replaced by saline. In preparations that are not so sensitive, such concentrations may produce only a rise in rate.

It has been observed that isolated arthropod hearts which have become fatigued or depressed are markedly restored by treatment with acetylcholine but that this restoration persists only as long as the drug is present. This is true of the heart of *Stenopelmatus*, the magnitude of concentration necessary to re-

esterase, physostigmine salicylate (eserine), has no discernible effect on the rate of heart beat in *Stenopelmatus* in low concentrations (10^{-5}); but treatment of the heart for 20 minutes to half an hour markedly lowers the threshold to perfused acetylcholine. Figure 1, *A*, shows an experiment in which the threshold was lowered to 10^{-7}. Figure 1, *B*, shows a test in which acetylcholine 10^{-6} greatly increased the rate and brought about transitory systolic tetany after eserinization, an action accomplished only by

much higher concentrations in the unpotentiated heart.

ACTION OF CARBAMINOYLCHOLINE (DORYL) AND ACETYL-β-METHYLCHOLINE (MECHOLYL)

These two choline esters have been shown by the writer (1942) to have a stimulatory effect on the isolated heart of the crab, *Cancer*, and by Garrey (*loc. cit.*) on the ganglion of *Limulus*. As was observed by Garrey on *Limulus*, doryl has a greater stimulatory effect on the heart of the insect *Stenopelmatus* than does mecholyl, the latter being unable to produce the extreme acceleration and rise in tone leading to systolic tetany that is characteristic of the former, acetylcholine, and nicotine.

ACTION OF NICOTINE

It is interesting that this alkaloid, known to have powerful stimulatory effects on ganglia, has in previous work been shown to act differently on the hearts of different insects. Hamilton (*loc. cit.*) found its effect to be largely inhibitory in the grasshopper, there being a slight and transitory increase in rate only at the highest concentrations used. Unfortunately, it is not made clear whether the paralysis induced by high concentrations was systolic or diastolic.

Yeager and Gahan (*loc. cit.*) noted that "in general, with regard to contraction rate, nicotine applied to the roach and larval heart preparations causes initial stimulation without depression at relatively low concentrations, initial stimulation followed by partial depression at relatively intermediate concentration, and stimulation followed by complete depression and paralysis at relatively high concentrations." They observed, however, that the roach heart was far more sensitive to nicotine than was the heart of the larval moth, *Prodenia*. Furthermore, they note that in the roach, "in those concentrations that gave marked initial stimulation of contraction rate, the quickened rhythm was usually accompanied by an obvious decrease in amplitude in the direction of incomplete relaxation of the cardiac walls, with the result that the rapidly beating heart remained more in systole than in diastole," and that still higher concentrations brought about *systolic* arrest. On the other hand, in the larval heart high concentration brought *diastolic* arrest.

In *Stenopelmatus* the action of nicotine is identical with the action described for the roach heart. Concentrations as high as 10^{-4} bring about a sudden rate increase and rise in tone leading to transitory systolic tetany, followed by depression in rate. Lower concentrations accelerate the heart without depression. This is also quite similar to the effects described on the crustacean heart (Plateau, 1880; Davenport, 1941) and on the ganglionic pacemaker of the *Limulus* heart (Carlson, 1906*b*, 1922).

The action of nicotine on the heart of *Stenopelmatus* is therefore similar to that of acetylcholine and doryl, except that the effects of the latter are more immediately reversible; this similarity of action prevents the observation of whatever blocking effects the alkaloid may have on the action of choline esters.

ACTION OF MUSCARINE

This alkaloid has been found to be stimulatory to the heart of *Cancer* (Davenport, 1942); but, in tests in which dilutions from 10^{-6} up to 10^{-3} were used, it was found to have no effect whatever on the heart rate of *Stenopelmatus*.

ACTION OF ADRENALIN

In the majority of the arthropods in which this drug has been tested (*Panulirus*, Welsh, 1939*b*; *Astacus*, Davenport,

Loomis, and Opler, 1940; *Carcinus*, Bain, 1929) and in the annelid *Lumbricus* (Prosser and Zimmerman, 1943), adrenalin has been found to have a stimulatory effect. Welsh noticed that often a sudden paralysis in diastole occurred after an initial great stimulation in rate.

Figure 3, *B*, shows that in the heart of *Stenopelmatus* adrenalin 10^{-6} inhibits rate reversibly and that higher concentrations (10^{-5}) bring the heart to almost

ACTION OF ATROPINE

The ability of low concentrations of atropine to decrease the rate of the arthropod heart slightly but constantly in both isolated and *in situ* preparations has been observed by many workers. Hamilton (*loc. cit.*) observed in the grasshopper that low concentrations "produced a slight chronotropic decrease, but the regular beat of the heart continued indefinitely at the lower frequency."

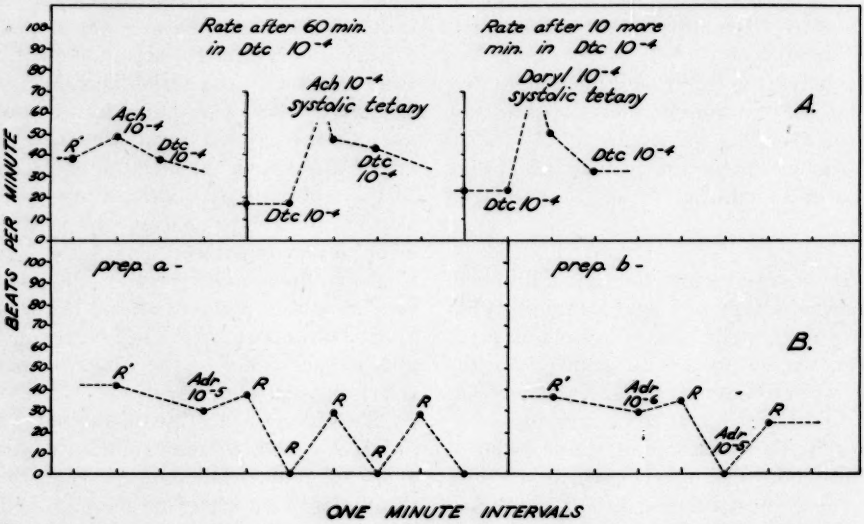

FIG. 3.—*R'*, base-line rate in saline; *R*, saline wash. Prep. A, action of acetylcholine and doryl after *d*-tubocurarine. Preps. B, action of adrenaline.

immediate diastolic arrest, this stoppage recurring periodically even after washing with saline.

Prosser (1942), working with the phyllopod crustacean *Artemia*, in which acetylcholine is without effect, attributes the apparent inhibitory effect of the Parke Davis preparation to the reducing agent, sodium bisulphite, present. In similar control tests on the heart of *Stenopelmatus* with sodium bisulphite alone, ranging in concentration from 10^{-5} to 10^{-3}, the inhibition produced by the Parke Davis adrenalin preparation could not be duplicated.

In *Stenopelmatus* the beat of the isolated heart under atropine 10^{-5} would continue for hours with a decreased but constant rate, resulting from an increase in the systolic interval. This rate decrease was accompanied by a marked increase in the total amplitude of beat, resulting from a lowering of tone.

EFFECT OF ATROPINE ON THE ACTION OF SYMPATHOMIMETICS AND NICOTINE

The effective and almost immediate block by atropine of the action of acetylcholine on the hearts of decapod Crus-

tacea has been observed by many workers (Maclean and Beznak, 1933; Welsh, 1939*b*, 1942; Davenport, Loomis, and Opler, 1940; Davenport, 1941; etc.). Of interest in this connection are Garrey's experiments (*loc. cit.*) in which he demonstrated, first, that acetylcholine is without effect in facilitating transmission of motor impulses to the musculature of the *Limulus* heart and later that atropine fails completely to abolish the stimulatory action of acetylcholine on the ganglionic pacemaker.

Hamilton (*loc. cit.*) demonstrated in the grasshopper that atropine "largely" abolished the effects of acetylcholine on the heart itself as well as on the alar muscles, which in his preparation were observed to contract under acetylcholine treatment.

In *Stenopelmatus* it was observed that, while atropine lessened the response of the heart to acetylcholine, it was rarely possible—and only after long perfusion with atropine—to demonstrate complete blockage (Fig. 2, *A*, *a*). Immediate blockage, such as that observed by Welsh in *Carcinus* and *Panulirus* (1939*b*, Fig. 1), could not be demonstrated (Fig. 2, *A*, *b*).

As has already been shown, the sensitivity of the unatropinized heart is least to mecholyl and greatest to doryl, with acetylcholine in an intermediate position. In the atropinized heart this is also the case; but the sensitivity to all three is apparently proportionately reduced, so that it is impossible to elicit a response to mecholyl, while the action of the other two drugs is but slightly "dampened" (Fig. 2, *B*).

In tests in which nicotine was administered after atropinization, no diminution of the response to nicotine occurred.

ACTION OF CURARE

Plateau (*loc. cit.*), testing the effects of curare on the intact heart of the crayfish, found that injection of 5 cc. at a concentration of 1:2,000 depressed both rate and amplitude but that only 1 cc. had no effect whatever on rate. Carlson (1906*b*, 1922), also working with commercial curare, found it to have initially a powerful stimulatory effect on the heart of the crab and the cardiac ganglion of *Limulus;* but he states that the potassium salts present in the preparation may have in part accounted for the stimulatory effect. At any rate, Davenport (1942), using curare Merck, was unable to demonstrate any acceleratory effect on the isolated heart of the crab *Cancer*, regardless of concentration.

In these experiments on the heart of the cricket, both curare Merck and the more powerful and chemically pure *d*-tubocurarine chloride Abbott were used. At very high concentrations (curare 10^{-2}, *d*-tubocurarine 3×10^{-3}), the two had an identical effect. With no increase in rate or increase in the diastolic interval, the beat, immediately after perfusion with the drug, became shorter as the tone of the heart increased, and paralysis in *systole* quickly occurred. This effect was reversible, and the beat quickly returned to normal after replacement of the drug by saline. Lower concentrations (curare 10^{-3}, *d*-tubocurarine 3×10^{-4}, 10^{-4}) were without immediate effect; but in some preparations depression of rate occurred after long perfusion.

EFFECT OF CURARIZATION ON THE ACTION OF ACETYLCHOLINE, DORYL AND NICOTINE

Katz (1936) demonstrated that curare fails to block motor impulses in the voluntary system of the crab; and the writer (1942) showed that curare abolishes the response of the crab heart to perfused acetylcholine.

In the heart of the insect *Stenopelmatus* it was found to be impossible to dem-

onstrate any clear abolition or "dampening" effects by subthreshold curare or *d*-tubocurarine on the action of subsequently administered acetylcholine, doryl, or nicotine. Figure 3, *A*, shows the effects of *d*-tubocurarine 10^{-4}, after which the characteristic acceleratory action of acetylcholine and doryl could still be elicited without diminution.

However, in a number of preparations, if depression of rate as a result of curarization had proceeded beyond a certain point, it was impossible to restore the normal beat even with high concentrations of acetylcholine or doryl, while nicotine, in spite of the curarization, would still elicit a powerful response.

ACTION OF DDT (2,2-BIS [P. CHLOROPHENYL]-1,1,1-TRICHLOROETHANE)

The resistance of the arthropod heart to DDT poisoning has been observed by other workers (Gordon, Crustacea, personal communication).

In tests in which the preparation was perfused with a DDT emulsion (5 per cent DDT in saline, peanut oil, mucilage of tragacanth) strong enough to elicit within 10 minutes the most violent spasms of the abdominal muscles, the heart continued beating strongly. Long perfusion with a suspension of DDT (100 p.p.m. made up from 1 per cent solution in ethanol) induced spasms in the abdominal muscles within 30 minutes, but the heart continued beating strongly for hours at a slightly reduced rate. Irregularity of beat appeared but could not be attributed to the direct effect of DDT on the heart, since the spasms in the abdominal muscles may have induced irregularity by altering the stretch of the heart or by mechanical stimulation.

Observations of the effect of DDT on the action of acetylcholine could not be made with this type of preparation because acetylcholine treatment further accentuated the complicating spasms of the abdominal muscles.

DISCUSSION

Tests with doryl, acetylcholine, and mecholyl were in accord with observations made on the hearts of other arthropods. The reason for the variation in sensitivity of different preparations to these drugs is not clear. The decreasing sensitivity to these drugs in the order listed may be explained by a differing esterase activity for them in the pacemaker of the heart, as has been observed by Tobias, Kollros, and Savit (1946) in roach nerve cord. Potentiation of the effects of perfused acetylcholine by eserinization gives evidence that the cardioacceleratory mechanism in insects may be cholinergic.

Prosser (1942) has suggested that hearts accelerated by acetylcholine are neurogenic, hearts inhibited are myogenic innervated, and hearts unaffected are noninnervated. Preliminary observations on the innervation of the *Stenopelmatus* heart have been made. By means of *intra vitam* staining with methylene blue, dissecting out the heart, staining with gold chloride, and teasing out the preparation, it has been possible to demonstrate ganglia and nerves closely invested to the heart muscle by the connective tissue surrounding it, The response of the cricket heart to the powerful ganglionic stimulant nicotine is identical with that of the heart of the roach (Yeager *et al.*, 1937, 1938), which is known to possess ganglia in lateral nerves (Alexandrowicz, 1926), and is unlike that of the larval moth *Prodenia*, in which McIndoo (1945) showed ganglia to be absent. The possession of closely associ-

ated ganglia and nerves and the similarity between the response to sympathomimetics and nicotine of the cricket heart and that of other arthropod hearts known to possess ganglionic pacemakers suggest that the beat of the heart of *Stenopelmatus*, in late instars at least, is neurogenic.

The actions of acetylcholine on different vertebrate tissues may be classified as muscarine-like, in which case they are abolished by atropine, or nicotine-like, in which case they are blocked by nicotine and curare. The actions of muscarine and acetylcholine on the cricket heart cannot be compared, since muscarine is without effect on it. The heart responds in a similar manner to both acetylcholine and nicotine, but the inability of both atropine and curare to abolish completely the effects of acetylcholine would suggest that it may not be possible to place the action of acetylcholine on the insect heart in either category.

Tobias, Kollros, and Savit (*loc. cit.*) demonstrated a rise in free acetylcholine in the roach central nervous system after prolonged DDT treatment. It had been hoped that it would be possible to demonstrate a lowering of the threshold for acetylcholine, explainable on the basis of a rise in pacemaker acetylcholine, after DDT; but this experiment can be attempted only in a heart preparation in which rate observations are not complicated by abdominal muscle spasms under DDT.

It is clear that further investigations are necessary, combining the effects of electrical stimulation of cardioaccelerator nerves according to the techniques of Carlson (1906*a*) or Steiner (1932) with the effects of subthreshold sympathomimetics, anticholinesterases, and blocking agents. Only in this way can a satisfactory picture of the mechanism of heart control in insects be obtained.

SUMMARY

1. Acetylcholine accelerates the heart of *Stenopelmatus* and causes transitory systolic tetany at high concentrations.

2. Eserine potentiates this action of acetylcholine.

3. Mecholyl, doryl, and nicotine accelerate the heart, the latter two having the more marked effect and causing transitory systolic tetany at high concentrations.

4. Muscarine is without effect on the heart.

5. Adrenalin is inhibitory to the heart and causes diastolic arrest at high concentrations.

6. Atropine abolishes the effect of mecholyl on the heart but only "dampens" the response of the heart to acetylcholine and doryl. The action of nicotine is unaltered by previous atropinization.

TABLE 1

COMPARATIVE DRUG EFFECTS

Mecholyl	+*
Acetylcholine	++
Doryl	+++
Nicotine	++++
Eserine	o
Acetylcholine after eserine	++++
Atropine	o, −
Mecholyl after atropine	o
Acetylcholine after atropine	+
Doryl after atropine	++
Nicotine after atropine	++++
Curare, *d*-tubocurarine	
high concentrations	Systolic arrest
low concentrations	o, −
Acetylcholine after *d*-tubocurarine	++
Doryl after *d*-tubocurarine	+++
Nicotine after *d*-tubocurarine	++++
Muscarine	o
Adrenalin	−, diastolic arrest
DDT	o
Acetylcholine after DDT	?

*+ = excitation; − = inhibition; o = no effect

7. Curare and *d*-tubocurarine are without immediate effect at low concentrations and at high concentrations cause systolic arrest without change in rate.

8. The response to acetylcholine, doryl, and nicotine is unaltered by previous treatment with curare and *d*-tubocurarine.

9. Suspensions or emulsions of DDT are without effect on the heart.

The comparative effects of drugs are presented in Table 1.

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DETECTION AND DISCRIMINATION OF ODORS OF AQUATIC PLANTS BY THE BLUNTNOSE MINNOW (HYBORHYNCHUS NOTATUS)[1]

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SINCE many fishes frequent the "weedy" habitat of lakes, it might be assumed that the aquatic vegetation plays some role in orientation. The object of this work was to ascertain if fish inhabiting the littoral zone of a lake were aware of the plants about them. To gain this objective, the olfactory sense was used as an indicator.

The aquatic habitat imposes severe limitations on vision because of turbidity and decreasing light with depth; moreover, many fish feed principally at night. It is not surprising that others who have gone before us discovered that difficulties in vision have been compensated for by specialization and improvement of the olfactory, gustatory, chemical, and tactile senses that are used in feeding, escape, and orientation.

To date, studies of natural odors (principally Strieck, 1924; Wrede, 1932; and Goez, 1941) have been confined to demonstrating that fish give off odors perceptible to their kind and that these odors help fish to aggregate. Also, the brilliant experiments of Von Frisch (1941) proved that the injured skin of the European minnow, *Phoxinus laevis*, gave off a substance (*Schreckstoff*), perceived through the olfactory epithelium, which initiated a fright response among its "school mates." It was this monumental work, in addition to the kind encouragement of its author, that gave impetus to a study of new sources of natural stimuli, namely, aquatic plants.

[1] Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. Professors H. F. Harlow and K. J. Arnold counseled us on testing techniques and statistical design; Mr. Warren Wisby gave liberally of his time to recording and processing data. We are indeed indebted to these colleagues.

METHODS AND MATERIALS

SOURCE AND CARE OF THE FISH

Bluntnose minnows, *Hyborhynchus notatus* (Raf.), raised in natural ponds in the University of Wisconsin Arboretum, were chosen. Intersexual variability was avoided by using males 70–80 mm. in length.

Bluntnose minnows had been used earlier by McDonald (1922) in a study in which he trained the bluntnose minnow to sound. He reported no deviations from standard aquarial procedures and was enthusiastic as to its suitability as a laboratory animal.

The advantages of using these animals were many. They proved to be hardy and required little space; a rectangular 7-gallon aquarium (17 × 11 × 12 inches), when aerated, supported eight adult minnows without crowding. Captivity appeared to have no distressing effect; and, when properly fed, second-year males remained in good health and rarely developed fungal infections. They did not require live food and lived well at room temperature, under which the tests were conducted. To avoid turbidity from excreta, the water was circulated and filtered through cotton.

BLINDING

The minnows were blinded to eliminate visual cueing during training periods and to reduce excitability. Blinding was accomplished by injection of a small quantity of phemerol into the posterior chamber of the eye. Within a few days degenerative processes culminated in a functionless sunken eyeball.

Surgery was conducted under 0.5 per cent urethane anesthesia. If fungus developed on the wound, one or two treatments of 5 per cent malachite green applied topically to the wound site were sufficient to control it.

In order to insure quick identification of each experimental minnow, a colored-glass embroidery bead was fastened to tantalum wire that had been sewn into the dorsal muscles of the fish immediately anterior to the dorsal fin. The flesh grew to the wire and, if well anchored, did not pull out. If it did, a distinctive scar was left which served to mark the fish.

DESIGN OF TANK

The essential problem in any discrimination test is to design a tank which agrees with the natural inclinations of the experimental animals. For this work a tank was designed which served as test container and living quarters combined. The addition of the odor was accomplished in such a manner as to keep out extraneous stimuli which might cue off the experimental fish. Figure 1 illustrates the mechanical features. Each experimental tank had a closed circulation, using an airlift principle (Innes, 1932), which also insured adequate aeration. This device was modified to insure circulation within the tank to allow introduced odors to reach the fish at a predictable time. Because the fish are bottom dwellers, the current was directed along the bottom; water was taken out on the surface. To spread the incoming water uniformly over the bottom, tiny jets released the water along the ends of the aquarium chosen for the feeding zone (for positive, stimulus) and punishment site (for negative stimulus). Halfway down the siphon tube was a T-drain, from which water was drawn to make up the odor rinse (not shown in Fig. 1). Air pressure for the lift was 15 pounds; when regulated by supply valves, it gave a flow of 0.10 ml. per second, a rate that insured complete circulation every 40 minutes.

The placement and separation of the circulations were tested by adding dyes (Fig. 2). The test substance was released through a 60-ml. separatory funnel, which emptied into the circulation. The emptying time was 65 seconds; no pressure differences occurred with this system. It took 20–35 seconds for the contents to issue from the jets—if the fish reacted sooner, it was assumed that they were responding to mechanical stimuli. Tests on persistence of aquatic plant odors showed that at initial strength no odor was perceptible after 20 hours. This property of the odor substances obviated changing water between tests. The manner of its removal is unknown.

Fish were trained to associate the end zones with reward and punishment. They were fed each time they reacted to the "positive plant" odor, which they would do by following up and orienting to the current gradient in the end zone. The negative punishment was an electrical shock (see below). The response was measured by (1) scoring the visits to the feeding area and (2) avoidance of the punishment zone after the introduction of the odor.

For administering the punishment, a set of two carbon electrodes, 2 inches apart, was cemented to the bottom in the end zone. The latter was smoothed over

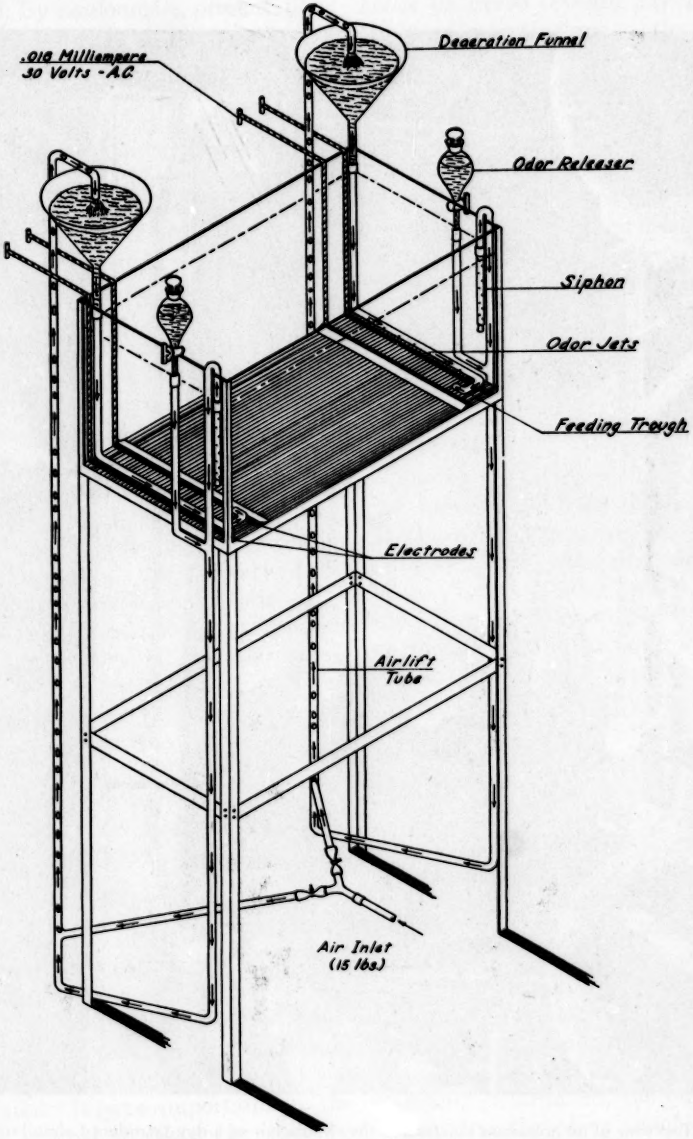

FIG. 1.—Design and construction of test tank

FIG. 2.—Top view of an aquarium illustrating the circulation of a dye introduced simultaneously from each jet.

by cement so that no serious barrier was presented. By randomizing presentation, the fish did not associate either end with any particular odor. The electrical system was a 110-volt, 60-cycle power line operating through a 600-volt transformer with a variable voltage input using a variac transformer. The secondary was connected in series to 17,000 ohms resistance and then sent to a switchboard, where it was connected to any particular set of electrodes in any tank by three-position, four-circuit, electrical snap switches. Microswitches were installed at each tank in series with the power supply. The voltage across the two electrodes was 30 volts; the current 0.016 milliamperes. Extreme care was exercised to shock fish only as they started into the zone rather than after they were in the zone. Furthermore, extensive shocking often made the fish wary to the extent that they avoided both stimuli.

The electrical field was restricted to about 1½ mm. beyond the electrodes. Thus fish associated the shock with the end zone and were not forewarned at a distance. In fact, when fish associated the odor with punishment, they swam just up to the electrodes, then backed away, swam well above it, or went directly to the other end.

FEEDING

A dry balanced diet, prepared according to the specifications of B. A. McLaren (1947), was used. Diet No. 41 was fortified with fresh liver and mixed in gelatin. It was then allowed to set on small perforated celluloid strips and was refrigerated. It was important in the training process to place the strip on which the food was bound in gelatin precisely in the end zone, so that the fish learned to expect food only there. To maintain motivation, the fish were not allowed to eat longer than 2 minutes. Such a rigorous routine led to malnutrition; hence on every seventh day they were fed heavily; testing was continued on the eighth day. On the day of rest the diet was also reinforced with live *Daphnia*.

CONDITION OF THE TEST GROUP

The minnows displayed extreme restlessness when kept alone; therefore, groups of six were settled upon as a convenient test unit. A larger group presented difficulties in scoring.

Two extra fish were maintained in each tank so that a trained fish was on hand in case of losses.

TREATMENT OF AQUATIC PLANTS USED AS A SOURCE OF ODOR

During the long periods, some in winter, required to train the fish, aquatic plants were maintained under strong illumination. If they deteriorated, new plants were collected locally, either in the University Bay of Lake Mendota or at the raceways of the State Fish Hatchery near Madison. Plants were washed and picked free of debris and animals. Each species of plant was kept separately in 5-gallon battery jars provided with running lake water.

The plants were at first removed each day just prior to the testing periods and placed in wide-mouth pint jars. This daily manipulation caused some crushing and breaking of plant tissues; and, as this was undesirable, the plants were carefully placed in the jars only once, and the jars were placed back in the stock battery jars at the end of the test period. The exact amount of plant was never carefully determined, the only criterion being that the plant should not be so crowded as to crush leaves in the bottom. The rinse water from such a small amount of plant had a scent sufficiently strong to provide, when rinsed, sufficient odor for the tests, even after three or

four washings. All species were pressed and entered in the Wisconsin Section of the University of Wisconsin Herbarium.

PREPARATION OF TEST RINSE CONTAINING THE ODOR

1. The jar with plant was drained.
2. The jar was filled with water from the siphon of the aquarium.
3. The lid was snapped on and the jar shaken vigorously twenty times, then the lid was removed.
4. This water was immediately poured through a cheesecloth filter into the separatory funnel. The rinse was used within an hour of preparation.

The separatory funnels were flushed with four rinses of water to insure that all the previous odor had been carried into the tank. Only then was the next odor added. The separatory funnels were drained and dried after every testing, to discourage algal growths which might have been different from those in the tank proper.

RECORDING OF DATA

Response of fish to the odor current was measured by recording which fish, after perceiving the scent, entered the feeding zone. A period of 45 seconds, subdivided into nine 5-second intervals, was used as a test period. The 5-second intervals were established in order to determine the approximate time that each fish spent in this area. To aid the recorder and observer in precise adherence to the fixed intervals, a timing device sounded every 5 seconds.

As each minnow entered the feeding area, his number was called to the recorder. A fish was scored as he entered the feeding zone in the interval between soundings of the timer. The boundary was set at 1 inch above the electrodes.

In addition, it was possible to observe and record the period elapsed between stimulus and reaction to the odor. This interval was designated "hesitation." The observer soon learned to recognize in trained minnows the difference between reaction and pretest behavior, which is not pronounced in untrained fish.

PROCEDURES AND RESULTS OF FIRST DISCRIMINATION TESTS

Training, when completed, answered the question as to whether the fish could discriminate between the odors from two different species of plants: positive behavior to scent of species A, negative behavior to scent of species B. The plan called for a group of sixty fish.

Ten tanks were divided into two lots of five; one group of five was assigned scent A as positive and scent B as negative; in the other group opposite assignments were made.

Each odor was presented once daily in the familiar ABBA scramble. The tests were done at irregular intervals. By this precaution fish were not conditioned to anticipate the test and food at a predetermined time of day.

A positive scent was followed by a feeding reward. The observer shocked the fish as they entered the end zone during exposure to the negative odor.

The total score was based on a mean for the thirty fish in the same lot. Once the positive curves reached the plateau, learning was considered as complete (Fig. 3). A second lot gave essentially the same result. This phase of operations was verified by running two 6-day periods of tests (extinction tests), which differed from previous training tests only by lacking reward or punishment. This eliminated secondary cueing, which resulted from the administration of shock. These values then represented the true state of differential learning, and the tests were stopped after 12 days.

Ten groups of six blinded fish each were trained to discriminate between

Myriophyllum exalbescens Fernald and *Ceratophyllum demersum* L. Scores for untrained fish are recorded in Figure 4. The progress of this learning can be seen from Figure 3, which is based solely on whether the fish scored, not on the time spent in the end zone. It shows clearly, however, the extent of learning for the negative odor.

It was of real interest and bearing on succeeding phases that each fish manifested his own ability to discriminate, instead of relying on the discriminatory powers of his tank mates. Some fish discriminated consistently with as little training as 1 month. A few never succeeded in learning, continuing to treat both odors positively. Still others developed a fear of both odors and would never enter the end zone during tests. These few were characterized by extreme nervousness, the slightest disturbance causing them to take flight to the surface.

PROCEDURE AND RESULTS IN DETERMINATION OF EXTENT OF TRAINING (PREGENERALIZATION)

"Pregeneralization" is the name given to the training immediately preceding

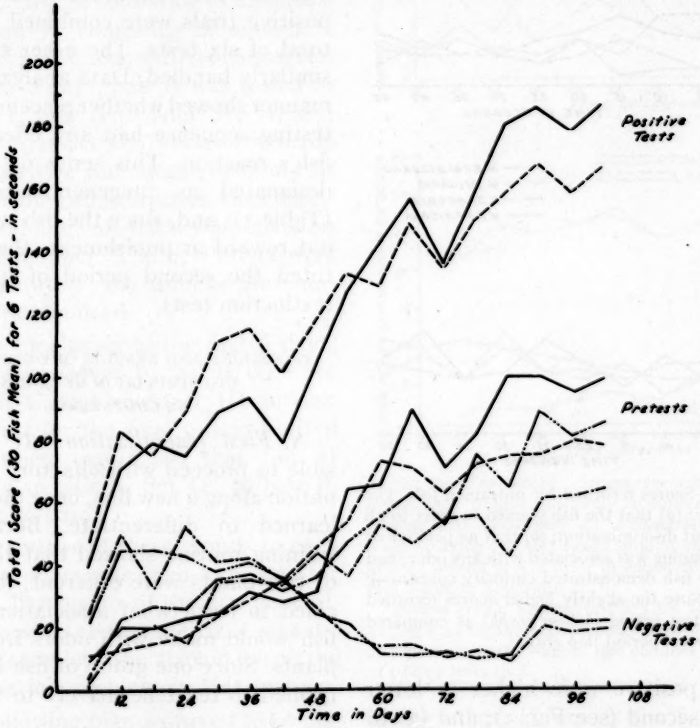

FIG. 3.—Composite record for two groups of fish during a 108-day period while establishing the conditioned response for two different species of aquatic plants.

Prelest: Scores recorded during a 45-second period prior to either the positive or negative test, i.e., random movement.

Positive test: Scores recorded during exposure to the odor which was always followed immediately by feeding.

Negative test. Scores recorded during exposure to the odor which was not followed by feeding—in fact, fish responding to this odor by entering the feeding zone received an electrical shock.

the generalization tests.[2] The objectives were (1) to discover whether or not fish would respond equally well to two positive and two negative tests in the same day and in the same testing period; (2) to discover whether the sequence in the testing affected the results, i.e., whether

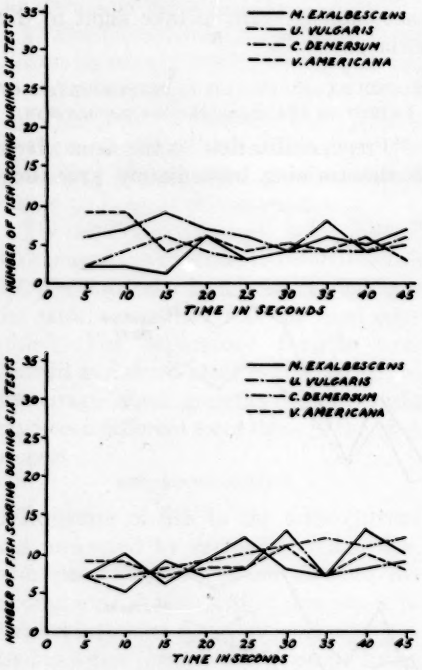
N. EXALBESCENS
U. VULGARIS
C. DEMERSUM
V. AMERICANA
NUMBER OF FISH SCORING DURING SIX TESTS
TIME IN SECONDS
0 5 10 15 20 25 30 35 40 45
0 5 10 15 20 25 30 35

FIG. 4.—Scores recorded for untrained fish. The results show: (*a*) that the fish seemed to react to all odors without discrimination; (*b*) that no positive or negative meaning was associated with any odor; and (*c*) that the fish demonstrated curiosity concerning the odors. Note the slightly higher scores recorded during the test period (*bottom graph*) as compared with the pretest period (*top graph*).

the first positive were higher or lower than the second (see Fig. 5); and (3) to determine the extent of the learning.

Once the fish had been trained ($2\frac{1}{2}$-month period) and this verified by making two extinction tests (tests of unlearning), the second and final generalization was begun. However, another slight variation in testing the extent of learning was made. After the first 6 days of the extinction test, the second period was completed with two positive and two negative tests, so as to put the fish on a four trials per day basis. In this phase all tests were carried out in one period, which commenced at the same time each evening to attain the same degree of motivation. The ABBA order of testing was again used. Trials for each succeeding day started with the next letter of the scramble; 4 days later it was initiated again.

In the analysis of these data all first positive trials were combined to give a total of six tests. The other tests were similarly handled. Data analyzed in this manner showed whether placement in the testing sequence had any effect on the fish's reaction. This series of tests was designated as pregeneralization tests (Table 1); and, since the fish were without reward or punishment, they constituted the second period of unlearning (extinction test).

PROCEDURE AND RESULTS OF DETERMINING DISCRIMINATION OF ODORS BY COMPARISON

A. *First generalization.*—It was possible to proceed with olfactory discrimination along a new line, once the fish had learned to differentiate. Because the training routine showed that the scents of two plants were different, it was decided to learn what association trained fish would make with odors from other plants. Since one group of fish had been trained to react negatively to the same

[2] Generalization test: To determine whether fish associate new odors with scents to which biological significance had been ascribed. It is a technique common to psychological experimenters, whereby the degree of similarity between two stimuli is determined by observing how nearly a second stimulus evokes the behavior associated with the first.

scent, odors from other plants, if similar, should induce similar reactions.

Because five pairs of tanks were available, three replicas of the same treatment were done, to see how consistently each group responded to the same two new stimuli. Accordingly, three sets of plants were selected, as follows: (I) *Anacharis canadensis* Michaux and *Ranunculus trichophyllus* Chaix; (II) *Vallisneria americana* Michaux and *Utricularia vulgaris* L.; and (III) *Potamogeton crispus* L. and *P. zosteriformis* Fernald. From the five pairs of aquaria, three were chosen for this work: pair A, consisting of aquaria 1 and 6; pair B, consisting of aquaria 2 and 7; and pair C, consisting of aquaria 5 and 8. For 6 days, pair A was tested to odor Set I, pair B to odor Set II, and pair C to odor Set III. Tests were made for the positive and negative odors as well as for the two new odors, thus maintaining training. All trials were made in the usual manner. The new odors were tested separately; the order of presentation was randomized.

At the conclusion of the first 6 days, odor Set I was given to pair B, odor Set II to pair C, and odor Set III to pair A. The test for the final 6 days shifted the treatment again, so that Set II was given to pair A, Set III to pair B, and Set I to pair C.

The reactions of three groups to each of the six odors was then available for analysis. The results of the first generalization were so clear cut that it was decided to complete the remainder of the discrimination problem in this manner.

The following plants proved to be dissimilar to *C. demersum* L. and *M. exalbescens* Fernald: *P. zosteriformis* Fernald, *P. crispus* L., *U. vulgaris* var. *americana* Gray, *V. americana* Michaux, *R. trichophyllus* Chaix. The only similarity was found in *A. canadensis* Michaux and this only with fish trained negatively to *C. demersum* L.

It was intended that, for two odors to be identical, the generalization should succeed in both tanks of the pair, namely, that the new odor should produce the positive reaction in one and the negative

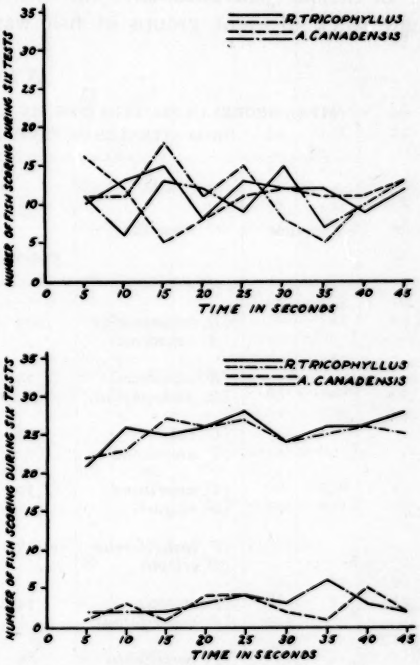

FIG. 5.—*Top graph:* Scores recorded for fish which were trained positively to *Ranunculus tricophyllus* and negatively to *Anacharis canadensis* while entering the test (end) zone of the aquarium in a 45-second period prior to exposure to the odor (see Table 1, Aquarium 1).

Bottom graph: Scores recorded for the same fish during a 45-second exposure to rinses of two species of aquatic plants. Double line signifies two groups of twelve tests each.

in the other. However, the fish reacted very strongly to the plant in question only when it appeared to have negative meaning. This suggested that the odor must be a mixture. Trained fish seemed to avoid any odor which had even slight similarity when punishment was the out-

come, whereas they required a much greater similarity to respond positively.

During the concluding weeks of testing the first generalization, it became evident that the fish had been damaged by what appeared to be a vitamin C deficiency.

B. *Second generalization.*—An entire new stock of eight groups of fish was trained, and the second series of the generalization tests was carried out. Reinforcement trials were made to maintain positive and negative training. These were carried out unrecorded on 2 out of every 3 days. To maintain motivation, the fish were not fed to satiation following the positive test except when it was the last day of the test series. When the positive test appeared elsewhere in the sequence, it was terminated by adding a few crumbs of food to the circulation. Such a slight feeding would, of course, have caused severe malnutrition; therefore, an additional positive test was given.

The results are presented in Table 2, indexed by pairs of aquaria.

Pair A, composed of aquaria 1 and 6, consisted of fish trained to discriminate between *R. trichophyllus* Chaix and *A. canadensis* Michaux. These were given as the fourth set to all the other groups as well. This was not necessary but was considered as a reaffirmation of the results of the first generalization.

Plants which failed to be associated with the training odors and thus were assumed to be different were *P. vaginatus*

TABLE 1

MEAN SCORES IN SECONDS (FOR SIX TESTS) OF TRAINED FISH REACTING TO THE ODOR STIMULUS OF VARIOUS SPECIES OF AQUATIC PLANTS*

Aquarium	Odor Set	Positive Odor			Negative Odor		
		Pretest	Test	Hesitation	Pretest	Test	Hesitation
1	*R. trichophyllus*	105	235	20			
	A. canadensis				99	23	155
6	*A. canadensis*	24	181	27			
	R. trichophyllus				33	19	201
2	*U. vulgaris*	108	188	18			
	V. americana				43	35	121
7	*V. americana*	30	178	19			
	U. vulgaris				26	35	122
5	*P. zosteriformis*	37	170	23			
	P. crispus				45	46	122
8	*P. crispus*	74	179	14			
	P. zosteriformis				60	32	150
9	*P. amplifolius*	89	156	32			
	P. vaginatus				64	30	148
10	*P. vaginatus*	78	200	11			
	P. amplifolius				50	29	179

* The negative odor was not accompanied by punishment.

TABLE 2

MEAN SCORES IN SECONDS (FOR SIX TESTS) OF TRAINED FISH RESPONDING TO THE ODOR STIMULUS OF VARIOUS SPECIES OF AQUATIC PLANTS

Aquarium*	Odor Set	Tests	Positive Odor†			Negative Odor‡			Generalization Test§		
			Pretest	Test	Hesitation	Pretest	Test	Hesitation	Pretest	Test	Hesitation
1	*R. trichophyllus*	1–24	78	193	13						
	A. canadensis					107	57	101			
	P. amplifolius								99	119	35
	P. vaginatus								79	109	56
6	*A. canadensis*	1–24	63	214	7						
	R. trichophyllus					57	46	127			
	P. amplifolius								67	98	50
	P. vaginatus								54	132	23
1	*R. trichophyllus*	25–48	84	229	2						
	A. canadensis					80	38	146			
	P. zosteriformis								77	124	28
	P. crispus								73	112	28
6	*A. canadensis*	25–48	71	219	5						
	R. trichophyllus					56	32	164			
	P. zosteriformis								63	130	29
	P. crispus								70	122	29
1	*R. trichophyllus*	49–72	75	207	9						
	A. canadensis					72	26	169			
	U. vulgaris								75	108	37
	V. americana								62	116	35
6	*A. canadensis*	49–72	75	221	6						
	R. trichophyllus					55	45	162			
	U. vulgaris								66	138	20
	V. americana								74	144	26
1	*R. trichophyllus*	73–96	83	199	12						
	A. canadensis					86	30	187			
	M. exalbescens								75	82	76
	C. demersum								62	57	133
6	*A. canadensis*	73–96	64	202	3						
	R. trichophyllus					49	26	115			
	M. exalbescens								54	137	34
	C. demersum								80	142	30
2	*U vulgaris*	1–24	73	193	12						
	V. americana					54	43	128			
	R. trichophyllus								73	135	28
	A. canadensis								72	130	22
7	*V. americana*	1–24	76	190	24						
	U. vulgaris					52	66	112			
	R. trichophyllus								66	124	41
	A. canadensis								68	158	21
2	*U. vulgaris*	25–48	68	220	6						
	V. americana					56	57	110			
	P. amplifolius								89	120	36
	P. vaginatus								85	118	27

* Aquariums: 1 and 6; 2 and 7; 5 and 8; 9 and 10 are pairs in which the plants to which fish were trained have opposite meaning.

† The odor with which fish had been trained to associate reward.

‡ The odor with which fish had been trained to associate punishment. No punishment accompanied the test.

§ Mean scores of trained fish reacting to odors of aquatic plants to which they had not been trained.

TABLE 2—*Continued*

Aquarium*	Odor Set	Tests	Positive Odor†			Negative Odor‡			Generalization Test§		
			Pretest	Test	Hesitation	Pretest	Test	Hesitation	Pretest	Test	Hesitation
7	*V. americana*	25–48	71	203	17						
	U. vulgaris					65	33	155			
	P. amplifolius								77	101	47
	P. vaginatus								72	86	52
2	*U. vulgaris*	49–72	64	193	12						
	V. americana					67	21	177			
	P. zosteriformis								92	78	73
	P. crispus								66	62	57
7	*V. americana*	49–72	50	203	12						
	U. vulgaris					41	22	185			
	P. zosteriformis								70	107	31
	P. crispus								44	115	56
2	*U. vulgaris*	73–96	86	198	8						
	V. americana					87	28	157			
	M. exalbescens								86	72	83
	C. demersum								56	62	65
7	*V. americana*	73–96	76	222	10						
	U. vulgaris					75	23	192			
	M. exalbescens								81	127	37
	C. demersum								64	166	55
5	*P. zosteriformis*	1–24	66	178	16						
	P. crispus					72	44	143			
	U. vulgaris								63	102	39
	V. americana								68	129	46
8	*P. crispus*	1–24	77	188	10						
	P. zosteriformis					62	50	131			
	U. vulgaris								52	129	22
	V. americana								57	116	39
5	*P. zosteriformis*	25–48	76	221	12						
	P. crispus					84	49	146			
	R. trichophyllus								85	165	24
	A. canadensis								77	180	14
8	*P. crispus*	25–48	70	229	2						
	P. zosteriformis					93	49	137			
	R. trichophyllus								79	154	23
	A. canadensis								76	139	21
5	*P. zosteriformis*	49–72	85	195	14						
	P. crispus					52	38	162			
	P. amplifolius								86	117	52
	P. vaginatus								77	124	47
8	*P. crispus*	49–72	82	197	11						
	P. zosteriformis					74	22	185			
	P. amplifolius								74	98	62
	P. vaginatus								79	52	109
5	*P. zosteriformis*	73–96	71	212	7						
	P. crispus					76	20	192			
	M. exalbescens								73	141	62
	C. demersum								84	153	38

TABLE 2—*Continued*

Aquarium*	Odor Set	Tests	Positive Odor†			Negative Odor‡			Generalization Test§		
			Pretest	Test	Hesitation	Pretest	Test	Hesitation	Pretest	Test	Hesitation
8	*P. crispus* *P. zosteriformis* *M. exalbescens* *C. demersum*	73–96	77	223	4	83	26	151	72 83	120 83	42 44
5	*P. zosteriformis* *P. crispus* *P. amplifolius* *P. vaginatus*	97–120	55	209	12	52	18	181	41 50	63 121	100 47
8	*P. crispus* *P. zosteriformis* *P. amplifolius* *P. vaginatus*	97–120	84	203	9	54	35	159	79 74	136 40	36 159
9	*P. amplifolius* *P. vaginatus* *P. zosteriformis* *P. crispus*	1–24	82	194	12	68	53	146	63 76	98 134	65 34
10	*P. vaginatus* *P. amplifolius* *P. zosteriformis* *P. crispus*	1–24	57	187	14	42	57	156	38 43	108 93	78 78
9	*P. amplifolius* *P. vaginatus* *U. vulgaris* *V. americana*	25–48	63	178	17	56	39	149	81 77	128 105	55 36
10	*P. vaginatus* *P. amplifolius* *U. vulgaris* *V. americana*	25–48	48	224	13	51	52	139	39 45	143 139	62 45
9	*P. amplifolius* *P. vaginatus* *R. trichophyllus* *A. canadensis*	49–72 (5 fish)	79	198	20	60	24	197	68 68	92 138	66 24
10	*P. vaginatus* *P. amplifolius* *R. trichophyllus* *A. canadensis*	49–72	62	196	11	42	34	161	62 58	148 139	37 44
9	*P. amplifolius* *P. vaginatus* *M. exalbescens* *C. demersum*	73–96 (5 fish)	85	157	28	69	21	180	52 77	67 97	74 79
10	*P. vaginatus* *P. amplifolius* *M. exalbescens* *C. demersum*	73–96	77	217	14	59	20	183	81 77	131 119	38 47

Tucz, *P. amplifolius* Tuckerm., *P. zosteriformis* Fernald, *P. crispus* L., *M. exalbescens* Fernald, *V. americana* Michaux, and *R. trichophyllus* Chaix. The plants which showed similarity were *C. demersum* L. and *A. canadensis* Michaux, but only with fish trained negatively to the latter.

Pair B, composed of aquaria 2 and 7, consisted of fish trained to discriminate between *V. americana* Michaux and *U. vulgaris* var. *americana* Gray. The following plants failed to be associated with the training odors and thus were assumed to be different: *R. trichophyllus* Chaix, *P. amplifolius* Tuckerm., *P. vaginatus* Tucz, *P. crispus* L., *C. demersum* L., *M. exalbescens* Fernald. A likeness was demonstrated for *A. canadensis* Michaux and *V. americana* Michaux, but only with fish trained positively to the latter. This was not repeated, however, and is hence of doubtful reliability, especially in the face of the fact that this combination was checked three times in testing the first generalization without similar results

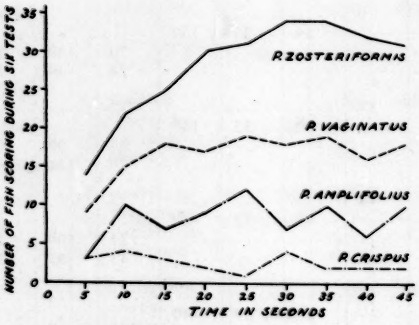

FIG. 6.—Scores recorded for fish trained positively to *Potamogeton zosteriformis* and negatively to *P. crispus*, showing that the fish did not generalize (associate), categorically, *P. vaginatus* or *P. amplifolius* with either (see Table 2, Aquarium 5, Tests 97–120).

Pair C, composed of aquaria 5 and 8, consisted of fish trained to discriminate between *P. zosteriformis* Fernald and *P. crispus L.* The following plants failed to be associated with the training odors and thus were assumed to be different: *V. americana* Michaux, *U. vulgaris* var. *americana* Gray, *R. trichophyllus* Chaix, *A. canadensis* Michaux, *P. amplifolius* Tuckerm., and *P. vaginatus* Tucz (Fig. 6). Slight association was observed in the following: *C. demersum* L. and *P. zosteriformis* Fernald, but only with fish trained negatively to the latter; and in *M. exalbescens* Fernald and *P. crispus* L., but only with fish trained negatively to the latter. A stronger likeness (Fig. 7) was observed for *P. vaginatus* Tucz and *P. zosteriformis* Fernald, but only with fish trained negatively to the latter.

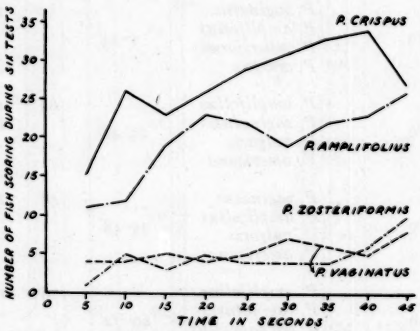

FIG. 7.—Scores recorded for fish trained positively to *Potamogeton crispus* and negatively to *P. zosteriformis*, showing that fish generalized *P. vaginatus* with *P. zosteriformis*. Doubtful generalization was shown for *P. amplifolius* with *P. crispus*. This generalization of *P. vaginatus* with *P. zosteriformis* occurred only with fish which had been trained negatively to *P. zosteriformis* (see Table 2, Aquarium 8, Tests 97–120).

Pair D, composed of aquaria 9 and 10, consisted of fish trained to discriminate between *P. vaginatus* Tucz and *P. amplifolius* Tuckerm. The following plants failed to be associated with the training odors and thus were assumed to be different: *U. vulgaris* var. *americana* Gray, *V.*

americana Michaux, *R. trichophyllus* Chaix., *M. exalbescens* ernald, *C. demersum* L., and *A. canadensis* Michaux. A likeness was demonstrated for *P. zosteriformis* Fernald and *P. vaginatus* Tucz, but only with fish trained negatively to the latter.

DISCRIMINATION OF ODORS BY OLFACTORY FATIGUE

Still another attempt was made to study discrimination based on the phenomenon of olfactory fatigue. Theoretically, to produce olfactory fatigue a continuous stimulus results in the blocking of the reception of more of the same stimulus and/or any other related stimulus. For this test previous training would not be imperative.

To saturate the aquarium with a scent, water was poured over a mass of loosely stacked plants. The previously cleaned plants were piled on a framed window screen placed over a circular wash tub; the water was poured over and over again for 20–30 minutes. So strong was the odor that to the human nose it resembled the fresh plant. The water was then passed through a cotton filter into the tank, after which the trained fish were returned.

Fish were then tested to see if they could detect the odor in the tank if more of the same were added. Other scents were prepared by drawing off aquarium water and shaking it over the new plants. The object here was to train fish to react indiscriminately to all plant odors in a positive manner. The test was scored in the usual way. At the conclusion of each test the fish were fed just a little food. In this way as many as a dozen tests were tried in quick succession without overfeeding, which might have destroyed motivation.

In practice, these studies were carried out with two tanks of fish. The fatigue odor was placed in one tank and the tests performed. The other tank was given the same dozen test odors but in normal aquarium water. These were called "prefatigue." Records were made of both these performances.

The next step was to replace the water in the prefatigue tank with newly prepared fatigue water and return the original water to the other group. In this way a second testing gave a replica of the original fatigue test; the first fatigue group was given tests called "postfatigue," which were handled in the same way as the prefatigue tests.

Two species of plants used to provide fatiguing water were *M. exalbescens* Fernald and *P. crispus* L. Data were obtained from fourteen separate tests performed over a period of 2 weeks.

Attempts were made to fatigue two tanks of six fish each. With *P. crispus* L. as fatiguing agent, the fish could not detect additional *P. crispus* L. (indicating that they were fatigued) but did react in each of the seven tests to *M. exalbescens* Fernald, *U. vulgaris* L. var. *americana* Gray, *V. americana* Michaux, *Chara excelsa*, *P. amplifolius* Tuckerm., *P. zosteriformis* Fernald, *P. richardsonii* (Benn) Rydb., *A. canadensis* Michaux, *C. demersum* L., *R. trichophyllus* Chaix, and *P. pectinatus* L.

Fish placed in water carrying the odor of *M. exalbescens* Fernald at a strength of 1:1,000, were not able to detect additions of the same odor, and they were assumed to be fatigued.

The following plant odors were detected by the fish seven out of seven times and therefore are presumed to have odors different from the one producing fatigue: *U. vulgaris* L. var. *americana* Gray, *V. americana* Michaux, *P. zosteriformis* Fernald, *P. pectinatus* L., *P. richardsonii* (Benn) Rydb., *P. amplifolius* Tuckerm., *P. crispus* L., *A. canadensis* Michaux,

C. demersum L., *R. trichophyllus* Chaix, and *C. excelsa*.

PERCEPTION SUBSTANCES IN PLANT RINSE BY OLFACTORY EPITHELIUM

After heat cautery of the olfactory tissue, fish trained to discriminate between *P. zosteriformis* Fernald and *P. crispus* L. were no longer able to detect these odors, much less discriminate. Of course, it had been suspected that these odors were perceived only by the olfactory sense because the fish were oblivious to the presence of odor when quiescent or moving downstream. Under these conditions probably not enough odor was brought into the nostrils to be detected.

If the fish had learned to discriminate these odors through taste, they still would have been able to do so after destruction of the nasal epithelium; but no reaction was observed. However, they did react to food particles placed in the water.

DETECTION OF MINIMUM CONCENTRATION OF ODOR RINSES

Measurement of strengths of odors was attempted, although it was realized that not only were the components of the odors unknown but the method of obtaining it by no means gave a uniform concentration from rinse to rinse. It goes without saying that, until the odor is purified and measured, the data will not reveal the true sensitivity of the fish.

All dilutions were introduced through the separatory funnel, which resulted in an additional dilution of 1:10,000. Further reduction in strength occurred at the region of the jets by the interaction of the odor stream with the adjoining water. If the rinses were so weak as to be near the threshold, the fish did not react to the odor because of the increased dilution at the jets.

Before making a dilution, the volumetric glassware had to be free of any stimuli to which the fish might react. To assure this, it was washed with water from the aquarium under consideration. If this water went undetected by the fish, the glassware was considered clean. The minnows were able to detect contaminants on glassware that had been cleaned chemically and rinsed many times. To circumvent this difficulty, the glassware was kept in the aquarium with the fish when not in use and was never cleaned again except to rinse it after the day's experiments.

DISCUSSION

Introduction of water rinses of aquatic plants invariably caused both trained and untrained bluntnose minnows to investigate energetically the areas of an aquarium into which the extract was streamed. This behavior permanently disappeared after the olfactory organs had been destroyed by heat cautery. It was therefore clear that the scents of the plant rinses were motivating the minnows' reactions. Furthermore, to rule out the possibility that vision was influencing their reactions, the fish were blinded by chemical cautery.

The odors from simple rinses of aquatic plants in this study were detected in a rinse streaming into a 7-gallon aquarium when diluted initially with 10,000 parts of water. This is a relative ratio only, because no quantitative figure was determinable for the amount of chemical compound (scent) rinsed from the plant leaves. Moreover, computations were not made on the dilution at the jets as the much diluted scent flowed into the aquarium. It goes without saying that the minnow's sense of smell is keen. In addition, the minnows could readily detect contaminants; in fact, new glassware had to be conditioned in aquarium water before being used.

One might postulate that plant odors

in nature from the myriads of plants filling the littoral zone of many lakes must yield amounts of odor sufficient to be distributed over considerable distances before excessive dilution occurred. The impermanence of these odors in the laboratory, however, suggests that such a tenuation might limit the extent of scent transport.

Training a group of fish to plant odors took 2½ months; therefore, the method of measuring discrimination of as many as twelve different species of plants in this study was made possible by a time-saving test known as the "generalization technique" (Hilgard and Marquis, 1940), i.e., a scheme to show likeness or unlikeness of new odors to a scent to which a fish had previously been conditioned by training. The advantages were: (1) it showed a relative degree of similarity of new plants to a plant to which the fish had been conditioned; (2) less time was required than would have been necessary by retraining the fish to the other combinations, requiring as many groups of fish as combinations. Moreover, if punishment were the outcome, the generalization tests helped to define only slight similarities of odors. Any likeness was associated with pain and gave a fright response. On the other hand, very great likenesses had to exist before the fish associated it with the positive stimulus.

Dissimilarity of odor was demonstrated by fatiguing the organs of smell to one scent, in order to ascertain whether others could be detected when added as superimposed odors. However, there were certain serious objections to these data. In the first place, it was not clearly established whether the fish were really fatigued or whether they had accommodated to a given concentration. It was not possible, however, to induce any response from stronger rinses. In the second place, the reliability of the data could be severely questioned if the odors used were mixtures. One might very well have effected fatigue to the principal constituent of the odor and yet still obtain a reaction if another odor happened to be washed off the plant, i.e., the odor of some animal. If one were dealing with a substance at known strengths and purity, the method and data would then be without objection.

Once fatigue of the olfactory organ has occurred, this should block detection of similar substances. In a few instances it was observed that fish under these conditions, assumed to produce fatigue, failed to react to a similar odor. The failure of animals in the last instance to react immediately indicated that they were, perhaps, no longer so sensitive to the odors as normally. An instance of undesirable fatiguing was to be seen in a tank which had not been cleaned for several weeks. Algal growths on the sides and bottom of the tank are sufficiently odoriferous materially to delay perception of various species of *Potamogeton*. The fish were unable to detect further additions of a strong odor rinse, but they could still distinguish scents from other plant species. Hence it seems unlikely that one plant odor will mask another. It may be, therefore, that a fish in nature can separate odors as it swims about, much as a dog follows a trail amid a distracting host of odors.

Although it was not possible to establish the chemical identity of the odors, preliminary steps toward purification indicated that the rinses were composites. This was as expected because nearly all aquatics have subterminal parts which are dying or dead, and on both are growing bacteria, fungi, periphyton, and invertebrate animals (*Aufwuchs*) which may be odoriferous in their own right. Plants used in the discrimination studies, however, were carefully selected to be

relatively free of these complicating features. Although highly desirable, it would have been a problem in itself to grow plants in a sterile environment.

It was not the primary intent to probe the social organization of the groups of fish. However, it was evident that the experiments had to be performed on groups because when solitary the fish were less steady. The individuality of the fish when confronted with an odor was clearly demonstrated, so that it was possible to appraise the extent of learning for each fish in the group. The fish behaved independently, showing little tendency to follow the leader.

Although punishment by electrical shock during stimulus with a negative odor made the fish slightly more belligerent, the bluntnose minnow, however, did not fight as did *Brachydanio rerio* (Hamilton and Buchanan), as reported by Breder and Halpern (1946).

In fishes most discrimination studies up to now have used the qualitative food-searching movements as the positive response. This has made it difficult to relate and compare data based on behavior. It is believed that the quantitative assays used in this study made it possible to compare at random the response to the stimulus with the response given during a contact period of equal length directly preceding the actual test.

Olfactory discrimination studies are normally carried out with known chemical compounds, which, although easy to obtain, probably never occur in the environment of the animal. Such stimuli permit precise measurement of olfactory thresholds, but little is learned about the stimuli occurring naturally in the fish's environment.

Because moist plants have a mild odor to man, one might suspect them to have some significance to fishes dwelling in the littoral zone, since aquatic plants are so conspicuous in the biotic community of that area. The results of our studies lend support to the view that aquatic plants may well play an important role in the life of a fish. They may serve as signposts to guide fish into feeding grounds, since many fishes commonly feed in turbid water, at dusk, at dawn, and at night, when visibility is poor. Moreover, plant odors may serve as attractants to prevent immature fishes from straying from cover and hence may be of significant survival value, much as the *Schreckstoff* from the skin of the minnow *Phoxinus laevis* (von Frisch, 1941). Perhaps, too, streams may have such characteristic odors as to guide migratory fishes, such as salmon and eel, to their homing areas. In addition, association of the plant odor by the fish to the food organisms inhabiting the plant may also be a possibility.

Apart from the natural olfactory stimuli affecting fishes, modern species which occupy habitats influenced by man's culture are often plagued with various irritating pollutants. No doubt, a fish's ability to survive depends in some measure on its capacity to sense the toxin in time to escape. Preliminary observations in this laboratory furnished evidence that fish can be trained to detect industrial wastes in extremely minute quantities. In fact, it may be possible to use minnows to assay some wastes biologically.

Since aquatic plants have specific odors, the possibility was suggested of using their scents in developing a system of classification. This work was not sufficiently extensive to suggest an arrangement of the genera of plants according to odor. Moreover, contaminating surface growths confounded the problem.

SUMMARY

1. Blinded, bluntnose minnows (*H. notatus* [Raf.]), in groups of six and eight, were able to discriminate, after a 2½-

month period of training, between the odors of *M. exalbescens* Fernald and *C. demersum* L. when food was the reward and electric shock was the punishment. Reverse training substantiated this.

2. Similarly, they were trained to discriminate odors of other sets of plants: (I) *R. tricophyllus* Chaix and *A. canadensis;* (II) *U. vulgaris* var. *americana* Gray and *V. americana* Michaux; (III) *P. zosteriformis* Fernald and *P. crispus* L.; (IV) *P. amplifolius* Tuckerm. and *P. vaginatus* Tucz.

Rinses containing odors of other plants were introduced into aquaria of fishes so trained in a Latin-square design by a generalization test. Of twelve species tested, the odors of only two seemed to resemble each other, i.e., when *C. demersum* L. had negative significance, the fish associated with it the odor of *A. canadensis* Michaux.

3. When minnows were fatigued to a strong rinse of one species, they still detected odors of other species as they were superimposed.

4. Trained minnows whose olfactory epithelium had been destroyed by heat cautery were unable to respond to plant rinses, thereby proving that the rinses contained a stimulating scent detected by sense of smell.

5. Minnows were able to detect a water rinse of a sprig of aquatic plant diluted 1:10,000 after it had been further diluted by streaming it into a 7-gallon aquarium. This demonstrates a very high degree of sensitivity.

6. Results of our studies lend support to the view that aquatic plants may well play an important role in the life of a fish. They may serve as signposts to guide fish into feeding grounds, since many fishes commonly feed in turbid water, at dusk, at dawn, and at night, when visibility is poor. Moreover, the odors of aquatic plants may serve as attractants to immature fishes to prevent them from straying from cover. In addition, other natural odors may direct migratory fishes in locating their homing areas.

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DOMINANCE IN WINTER FLOCKS OF JUNCOS AND TREE SPARROWS

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THE social behavior of a flock of wild birds will naturally be studied in the light of laboratory findings on groups of domestic and captive birds (Collias, 1944). These suggest that a wild flock may resemble a captive group in its organization and be subject to some sort of pecking order (Guhl and Allee, 1939). Studies of chickadees by Odum (1942) and Hamerstrom (1942) bear out these suggestions. It has also been conjectured, however (Lorenz, 1935), that the pecking orders among domestic fowls and captive groups of the laboratory are artifacts and that it may accordingly be doubted whether such a social organization exists in nature among "unsocial" birds.

Juncos and tree sparrows are unsocial in Lorenz's sense of the word, since they do not hold territory in common or breed in colonies. Do they then lack social organization in the winter flock? If not, in what pattern are they organized? Is there a hierarchy in respect to dominance, such as Schjelderup-Ebbe (1935) considered typical, or is there organization in some other recognizable pattern, such as the peck-dominance relation described as existing among pigeons by Masure and Allee (1934)? Does the fall migration bring to the wintering area a group of completely unorganized birds, so that the observer is watching organization emerge out of a disorganized state? It was with questions such as these in mind that the observations reported here were undertaken.

MATERIALS AND METHOD

Two series of observations were made. The first series was made in 1947 of a flock of fourteen slate-colored juncos (*Junco hyemalis hyemalis*) which visited a feeding station at the observer's home. The second series, in 1948, was made of a flock of eleven juncos and a flock of six tree sparrows (*Spizella arborea*). All birds were conspicuously marked by colored feathers cemented to the upper pair of tail feathers.[1] By this means they were easily and quickly distinguishable as individuals.

The point of observation was an expanse of glass doors looking toward the woods from which the birds came. The necessary cover and perching places were provided by setting up a dense artificial hedge of felled evergreens and bare branches to inclose a shallow arc of space outside the glass. The observer's chair was partly hidden behind the solid wall. So alert were the birds to motion within the room that only extremely guarded movements were possible. Dark clothing was essential, and a box was placed on the writing table to shield the movements of the right hand.

There were several problems in the management of the feeding station. It

[1] The birds in the 1947 series had been color-marked by M. J. Westfall, of Cornell University, for purposes connected with his own work. The writer wishes to express her gratitude for his encouragement and for the use of his banding records. Dr. Westfall is now associate professor of biology at the University of Florida. The writer is indebted to Professor W. A. Wimsatt for two post-mortem examinations.

was important for the sake of easy visibility to have the feeding area well above the ground and to keep the food and the birds off the ground. Canary-grass seeds were found to be the most suitable food, since juncos and tree sparrows eat them readily and they do not attract chickadees, nuthatches, blue jays, or squirrels. The seeds were so light, however, that even the fanning of the birds' wings in taking off scattered them. It was also important to keep food available during snowstorms, since it was then that the largest numbers of birds visited the station. Another problem was how to provide ample feeding space and yet be able to decrease the size of it in order to encourage competition during observations. Finally, it was desirable to have a means of estimating reliably the distances between the birds, in order to measure degrees of feeding tolerance. The most satisfactory method found was to place a bench against the hedge and offer the food in shallow wooden trays, 1 foot square or 1 × 1½ feet, placed on the bench.

The observer was from 4 to 7 feet from birds in the main center of activity on the bench. In February a trapping cage was set up at the end of the inclosed area in an attempt to catch house sparrows. The birds used the trap as a feeding place when it was open and baited and were then about 10 feet from the observer.

The conditions most favorable to bringing the birds to the station were snow-covered ground, combined with snowfall or rain. Conditions during March and April, 1947, were severe for the time of year but unfavorable when compared with January and February, 1948. As a consequence, 300 hours were spent on the 1947 series, while the 1948 observations were completed in 184 hours. The latter were terminated by the disappearance of snow on March 11.

It is desirable to know whether rank in the hierarchy and sex are related. Neither juncos nor tree sparrows, however, are distinguishable as to sex. An apparent sexual dimorphism of the junco, in the form of a brown and buff suffusion which often modifies its slatiness, is misleading. It "occurs more frequently and more extensively in females than in males, but it is also clear that no complete sexual difference exists "(Miller, 1941, p. 315).[2] A similar uncertainty exists in identifying the sex of tree sparrows (Heydweiller, 1935).

OBSERVATIONS

CRITERIA OF DOMINANCE

The mark of reciprocal dominance and subordination was taken to be (with both species) the ability of a bird to stimulate a retreating reaction in another bird. This ability was shown most clearly through four patterns of behavior, and these four, therefore, were the standard events which were entered in the charts. No event was counted unless it involved a clear case of contact and of retreat on the part of one of the birds.

1. The peck, with retreat as the response, was the most frequent of the aggressive moves by which one bird displaced another. The pecks were gestures, in the sense that they did not "land" on the retreating bird. They varied in speed and intensity from a mere thrust of the bill in the direction of the other to a vigorous run or flight of enough pace and momentum to carry the aggressive bird up to the other. Retreating reactions also varied from a recoil of a few inches to a

[2] The greater the amount of brown and buff, the lighter the birds appear. The observer arranged 38 junco specimens in the Cornell University collection in three groups of dark, intermediate, and light, corresponding to the distinctions attempted between the birds in the field. The distribution as to sex turned out to be as follows: 12 dark, 10 males and 2 females; 18 intermediate, 12 males and 6 females; 8 light, 1 male and 7 females.

run, a flight away or, occasionally, a flight straight up. About 82 per cent of the chart figures are based on events of this type.

2. When two birds found themselves suddenly close together, one might avoid the other instantly and with some violence. A bird about to alight, for example, might hover with swirling wings in order to avoid alighting near another bird. Or one bird might take off instantly at the sudden approach of another. These reactions were accepted as marks of subordination.

3. Threat postures were regarded as evidence of dominance if they were exhibited by both birds and terminated in the prompt retreat of one. In threat postures, the junco draws the body up, throws the head up repeatedly, keeps the bill closed, and creates the effect of added height. The tree sparrow reaches toward the opposed bird with fanned tail and open bill.

4. Fights varied from brief flutters close to the ground to flights straight up, opponents facing, for 10 or 12 feet. No struggle on the ground took place after the upward flight. The two species were similar in their fighting. There was no pecking during a junco fight, but this point is not certain in the case of the tree sparrow. To be accepted as evidence of dominance, a fight had to terminate either in the flight of one bird from the station or in the immediate return of one (the victorious bird) to the feeding place where the fight started. Fights which ended in the two birds eating together or perching were not regarded as conclusive in respect to dominance and were not entered in the charts. One fight was entered in Chart I (the only fight observed in 1947); four were entered in Chart II out of eighteen observed; no fight was entered in Chart III.

EXPLANATION OF CHARTS I, II, AND III

The horizontal row at the top of each chart gives the names[3] of the individual birds arranged in their linear rank with the alpha bird at the left. The vertical column at the left is the same list with the alpha bird at the top. The chart is to be read from left to right: B had no contact with RB, displaced CL three times, WL twice, etc. (examples refer to Chart I).

Each chart is twofold. The figures above the diagonal on the right give the frequencies of the pair-contacts on which the ranking of the birds is based. Those in the lower left record all pair-contacts not in accord with that ranking. Thus, if two birds peck both ways, e.g., if A pecks B and B pecks A, there is a square available for each event. The double chart shows, therefore, the number of pair-contacts and relations that are in a hierarchical line and those contrary to it.

Any two squares referring to the same two birds lie diagonally opposite each other and at equal distances from the diagonal line. This relation is readily seen in the two squares: B pecked RB, RB pecked B. It will be convenient to refer to the contacts in the upper right as "regular" and to those in the lower left as "irregular."

The irregular pair-contacts (i.e., those below the diagonal) fall into three groups: (1) Reverse pecks: for example, GY pecked CR three times and is dominant to CR in the ranking. But CR pecked GY once. These reverse pecks are marked with an asterisk. (2) Triangles: for example, CW pecked GB three times but is below GB in the ranking. GB did not peck CW. These irregular contacts are marked with a triangle. (3) Inconclusive pecks: no symbol is attached to a single

[3] Bird names are not symbols of rank. They are derived from the colored feathers; i.e., *B* means blue, *L* means brown, etc.

irregular peck if there were no regular pecks between the birds. Further contacts were needed to make clear whether these pecks were reverse pecks, as under group 1, or triangles, as under group 2.

DOMINANCE IN THE 1947 JUNCO FLOCK

The fourteen birds in the 1947 flock were described by Westfall (1947) as one of three junco flocks wintering in the woods near the observer's feeding station. Forty juncos, believed to include practically the entire junco population of the 9-acre tract, were trapped and marked by Westfall in February and appeared to form three flocks. Though they separated into flocks to forage, they had an "area of convergence" about Westfall's feeding and trapping station in the woods. One of these flocks fed at a station on the northern border of the woods. (The observer's station was at the southwestern edge of the tract and about a quarter of a mile from the station just mentioned.) So far as is known, the third flock foraged in the woods. There was some casual visiting by individuals from flock to flock. The observer saw, all told, eleven casual visitors, three of which, on a very stormy day, ate and had pecking contacts with the regular flock. The casuals evoked no fighting or hostile display from the regular flock. Of these three casuals, one was completely subordinate; one pecked the two lowest birds in the regular flock and was pecked by others; and the third was pecked twice by GY but pecked WL twice, thus creating a triangle. These casual visitors formed one class of apparent strangers.

Another group of strangers was made up of the migrants that appeared in April. It should be noted that when these birds appeared the resident birds were dominant over them. Three resident birds—WL, GY, and WW—still appeared at the station and ate frequently with considerable numbers of migrants (occasionally as many as fourteen). Even WW, next to lowest bird of the resident flock, was, almost without exception, dominant to the migrants and when with them had the confident air often seen in the alpha bird, B.

Chart I presents the data on dominance in the 1947 junco flock.

The relatively large number of pair-relations above the diagonal, compared with the number below, creates a reasonable presumption that the birds dominated one another in a linear hierarchy, with the exception of one relation which created triangles. (Reasons for not regarding the four single irregular contacts as creating triangles were given above under the explanation of the charts.) CW's dominance over GB produced three triangles, among the two birds and LR, GY, and Y, respectively. CW and LR were in a position of apparent equality in Chart I. In 1948 these two birds returned, and LR was then the dominant.

There are, however, thirty-three blanks which might, if filled, show so many contradictory pair-relations as to throw doubt on the conclusion that there was a straight-line pecking order. The distribution of the thirty-three blanks requires comment. Twenty-three blanks out of the thirty-three are distributed among all the possible contacts of the four intermediately ranked birds—YB, Y, CW, and CR. GY was dominant to all these birds, and the five appeared together as a group several times. Among themselves the four birds have a linear order (with 2 blanks). The other ten birds also, regarded by themselves, have a linear order, with ten blanks out of forty-five possible pair-relations. Twenty-one blanks, therefore, are distributed among the relations of the four with the ten. The twenty-one blanks are distributed as follows. There

were twenty-four possible relations of the four birds with the six ranking birds, of which thirteen were blanks; and the four birds also had twelve possible contacts with the three subordinate birds, of which eight were blanks. It was possible in 1947 for birds to avoid pecking contacts by eating on the ground because a good deal of food was scattered on the snow during January and February (before observations were begun) and was exposed by successive thaws. CR adopted this behavior conspicuously and was recorded as a "cautious" feeder. The fact that twenty-one out of thirty-three blanks occurred in the contacts of four closely ranked birds suggests that these birds may have formed a subgroup, partly isolated by avoidance from contact with other members of the flock but completely dominated by GY.

ANALYSIS OF CHART I

Regular pair-relations	51
Regular pair-relations including reverse pecks	2
Pair-relations creating three triangles	1
Inconclusive single irregular contacts	4
Complete blanks	33
Possible pair-relations of fourteen birds	91

CHART I

MARCH 3 - APRIL 20, 1947

JUNCOS

	B	RB	CL	WL	GB	LR	GY	YB	Y	CW	CR	YC	WW	WG
B			3	2	2	2			1			5	2	4
RB				1	1	4				2		4		1
CL				2			1		1				1	
WL					1	8	5				1	2	9	1
GB						4	4		4	Δ		1	1	1
LR							5			1		2	4	2
GY								1	9	5	3	4	5	1
YB		1							3		1	1		
Y						1				2	3		1	1
CW					3Δ	1✕						1		
CR	1	1					1✕							
YC													2	
WW														2
WG														

DOMINANCE IN THE 1948 JUNCO FLOCK

Inspection of Charts I and II shows that two birds of the 1947 flock, LR and CW, returned in 1948. A third, CL, also returned but is not included in Chart II because of its accidental death after one day with the flock. On two occasions a banded bird of the 1947 population also returned as a casual visitor to the observer's station, and at least three visited the station north of the woods. Hence it may be stated that, of the forty juncos banded by Westfall, at least six and possibly eight returned. Since in 1948 only the birds that visited the observer's station were marked, nothing is known about the winter population in 1948 and its division into flocks.

The sex of two members of the 1948 flock is known. Post-mortem examination showed that O and SO were males. YG, the omega junco, was extremely small and light colored, and CW was extremely dark.

There was little casual visiting in 1948. In all, only three casual visitors were seen. These three apparent strangers were subordinate in all their contacts with the regular members of the flock.

SO, OC, BR, and YG remained during the spring migration and were dominant to the migrants, as had been the case with resident birds in 1947. With one exception in the case of YG, no migrant was seen to peck a resident bird. Chart II gives the data on dominance in the 1948 junco flock.

The presumption of a linear hierarchy is obviously much stronger in Chart II than in Chart I. One pair-relation in Chart II, as in Chart I, creates triangles; SO's dominance over CW creates triangular relations with OC, R, and RL. It may be noted that CW gave one reverse peck to SO, as other subordinates occasionally do.

The number of blanks is small enough to render uncertainty from this source much less than in 1947, when the regular pair-relations were only 58 per cent of the possible number, while in 1948 they were 87 per cent.

Of the juncos in the 1948 flock, the eight which were first trapped appeared from the start of the observations to be an assimilated group. Four other birds (RL, YG, BR, and CL) were trapped later, though not together, and the behavior of these birds calls for special description. For 8 days RL avoided the other birds, making only four contacts, and sought for food on the ground rather than at the trays, even though it might be alone at the station. On the ninth day RL suddenly pecked NR, and its behavior changed. It began to eat on the bench and to exchange pecks with the eight original birds. BR, which was trapped still later, behaved in much the same way as RL, except that the period of avoidance lasted for only 3 days. YG, on the contrary, seemed to be completely integrated from its first appearance. It ate from the trays, showed no reactions of avoidance, and on its first day made contacts with every bird in the flock except NR. CL, the bird not included in Chart II because of its accidental death, was in the flock only a single day. On this one day, however, it had successful fights with O, LR, and R, and submitted to several pecks (with one reverse peck) from RY, the alpha junco. Its indicated position as the beta bird was consistent with its position in the 1947 flock (Chart I). CL's behavior appeared to be of the sort that would be expected from a dominant, aggressive bird making initial contacts in an unfamiliar flock. It is possible that RL, BR, and CL should be regarded as strangers joining the flock.

A feature of junco behavior (in both years) was the variation between indi-

viduals in the frequency of their visits to the station. Chart II shows, for example, that NR and RL have low figures in both their horizontal rows and their vertical columns. The explanation of the small numbers of contacts lies in the fact that they made fewer visits. O was the next most infrequent visitor to the station. In general, large numbers of contacts accompanied large numbers of visits.

ANALYSIS OF CHART II

Regular pair-relations	42
Regular pair-relations including reverse pecks	6
Pair-relations creating three triangles	1
Inconclusive single irregular contacts	2
Complete blanks	4
Possible pair-relations of eleven birds	55

BEHAVIOR PATTERNS ASSOCIATED WITH DOMINANCE IN THE 1948 JUNCO FLOCK

The four distinct patterns of behavior described earlier and recorded in the charts were accompanied by many other patterns closely related to the pecking order. These latter were not of a sort to be proof of dominance. Rather, they were understandable only in the light of a pecking order already known.

CHART II

JANUARY 3-MARCH 11, 1948

JUNCOS

	RY	O	LR	CW	OC	R	RL	NR	SO	BR	YG
RY		9	10	11	9	23	4	1	8	9	13
O			10	9	5	3	1		3	2	
LR	1[x]			15	21	28	2	1	5	16	2
CW					43	38	5		1[Δ]	5	7
OC						21	13	2	16	25	16
R			2[x]				13		17	23	33
RL				1[x]				2	2		1
NR				1					3	1	
SO	1[x]			20[Δ]	1[x]	1[x]				3	10
BR							1				42
YG											

Patterns of avoidance.—(*a*) A subordinate bird may avoid one dominant to it by quiet movements away from the latter's vicinity. Movements of this sort were counted during January, 1948; there were 76 cases, 67 of which ran parallel to the relations of the birds in the pecking order. (*b*) A dominant bird perching in the hedge a foot or so above a subordinate bird which is eating constitutes a threat; the subordinate hops restlessly, eats faster, and sometimes leaves. (*c*) If a dominant is eating in a trap-cage, a subordinate does not enter. If a subordinate is eating in the cage, a dominant may enter. The subordinate leaves at once, even though not pecked. If the dominant bird happens to block the exit by standing in it, the subordinate shows marked, even frantic, escape reactions in trying to find a way out. (*d*) When a subordinate bird perches and sees dominant birds on the bench, it may go to the ground or wait or fly back and forth from hedge to ground or creep slowly through the hedge, hop on the bench, and hover on the outskirts. Occasionally, such a bird darts in, snatches one seed, and darts out. Or it starts to eat but sidles and backs while doing so. (*e*) The low-ranked birds fly in to eat alone far oftener than others do.

Indifferent tolerance.—It is possible to distinguish two types of tolerance, describable as "indifferent" tolerance and "hostile" tolerance. Indifferent tolerance applies to the behavior of a dominant bird which permits a subordinate to feed within 12 inches of itself for 2 minutes or more. (These measures were selected as marking unusual behavior.) The distance may be 4–6 inches and the time about 10 minutes. A peck may terminate a period of tolerant feeding. In any case, the subordinate nearly always leaves first. Sixty-nine instances of indifferent tolerance were recorded. The lower birds in the ranking order do not often tolerate still lower subordinates. In 65 cases the dominant was one of the five high-ranked birds. The two subordinates which were tolerated most often were R (50 per cent of the cases) and YG (20 per cent).

Hostile tolerance.—This is a procedure of resistance initiated by the subordinate bird, which approaches a feeding dominant with threatening postures of varying intensity (throwing up the head, stretching up). The dominant bird responds with similar gestures. They eat together, picking up a few seeds and posturing alternately. This pattern may shade off into indifferent tolerance. Occasionally, the initial hostility of the subordinate is evoked by a dominant which approaches it as it eats. If the dominant does not peck but responds with counter-posturing, they eat together as described above. As in the case of indifferent tolerance the distribution of this behavior in respect to subordinates was strikingly uneven. R was the initiating subordinate in 62 per cent of the sixty-one instances observed, and YG in 26 per cent. The dominants were the high-ranked birds in fifty-six cases. These facts suggest that, for certain birds, hostile tolerance diminished the rigor with which the pecking order restricted their access to food. They also indicate that birds differed considerably in their employment of the possible patterns of behavior; R and YG were outstanding in using hostility to gain tolerance from the five high-ranked birds.

Resisted pecks.—A period of hostile tolerance, with its alternate posturing and eating, might last for several minutes, and it was usually brought to an end by the subordinate's leaving first. It might also end with a peck which drove the subordinate away. There was, however, a further possibility: the subordinate might resist the peck. Resistance took the form of posturing vigorously and

remaining on the spot, with no retreat by either bird. There were then two alternative reactions by the dominant bird. It might "acquiesce," and the two birds would then continue to eat together, or a fight might ensue.

There were nine cases in which a subordinate successfully resisted a peck by a dominant bird under the circumstances described. In two cases R resisted a peck delivered by OC. The seven other pecks of this type were all resisted by YG, the omega junco. Three of these were delivered by LR in a single period of hostile eating. Each time that LR pecked, YG threatened, and the upshot was that YG continued to eat with LR and finally left without being pecked again. In addition to these three consecutive pecks, on two other occasions YG resisted single pecks from LR. Chart II shows that LR pecked YG only twice; in addition, however, there were these five cases in which YG successfully resisted a peck by LR. The chart shows also that O did not peck YG. As a rule, complete blanks on the charts mean that no contact was observed between the two birds involved. This is not true as between O and YG. O was seen to tolerate YG four times; in one of these cases O finally delivered a peck, YG resisted and remained to eat. The seventh peck resisted by YG was from OC, a bird which pecked YG sixteen times in the course of the observations. YG's ability to resist pecks from three high-ranked birds is a case of marked individuality in behavior.

Fights for tolerance.—It was remarked above that resistance to a peck might end in a fight. There were four fights that were clearly identifiable as having originated in this way. Two of the four ended by the birds eating together. Since this result, that the subordinate ate with the dominant, was the same as the result of the posturing, or of the posturing followed by the resisted peck, these fights should probably be considered as the final link in a chain of procedures by which a subordinate bird might resist a dominant and even win a fight. It was not true, however, that the subordinate always won a fight of this kind. In the other two clear cases of fights for tolerance the resisting subordinate left the station and once was chased vigorously for several yards.

There were fourteen other fights between juncos in which the outcome was seen and the combatants were identified but the preliminaries to the fight were not seen. Either the beginning was not seen at all, or there were no hostile preliminaries, or they were too brief to be analyzed. Twelve of the fourteen ended merely by the birds perching. It is not possible to say whether these inconclusive fights belong to the class of fighting for tolerance in feeding or to the class of aggressive fighting to establish status in the hierarchy. The two remaining fights, which were conclusive, resulted in no change of status. Hence the nature of these also is open to question.

In the total of eighteen fights mentioned above, it is notable that R was a participant in fourteen and was the subordinate bird in all four of those which clearly began with hostile eating and resisted pecks. R's record is therefore distinctive in respect to the employment of the two types of tolerance and the procedure of resistance arising out of tolerance, and also in respect to the number of fights in which R took part.

Patterns of dominance.—(*a*) A special quality of behavior was often discernible in the alpha bird and in subordinate birds relative to it. The alpha bird was quick and decisive in pecking to clear the board; its behavior might be described as conveying an air of confidence. On the other hand, there was a tendency for the

other birds to keep their distance, once the dominant had established itself at the feeding tray. B, the alpha bird in 1947, showed this distinctive pattern of dominant behavior, and GY had it when alone with the four birds immediately subordinate. RY and O, in 1948, were decisive in action and usually ate in isolation, which made pecking unnecessary. So characteristic was this quality of behavior that the leading birds could be selected even before the pecking order was known, if they appeared sufficiently often. (*b*) Dominant birds varied considerably in the frequency with which they displayed the associated patterns of dominant behavior. In respect to indifferent tolerance, O showed the greatest number of cases and RY the smallest. In respect to hostile tolerance the order of the two birds was reversed: RY participated in many cases and O in few. If both kinds of tolerance are counted, LR was the most tolerant of all. OC was the only bird that frequently tolerated its subordinates and was also frequently tolerated by its superiors.

Reverse and inconclusive pecks.—These irregular pecks that did not conclusively make triangles were alike in one respect: they occurred only in periods when the birds were eating rapidly under the tension of competition. In general, all birds showed a marked tendency to eat faster when other birds approached. In the case of juncos the rate of feeding (measured by the number of seeds picked up per minute) of a bird eating in solitude, as compared with the same bird continuing to eat after other birds had alighted near it, might be speeded up by anything from 50 to 100 per cent. Under stimulating weather conditions, when numbers of birds were feeding at the same time in a restricted area, rapid sequences of pecks and retreats and returns by subordinates after being pecked were the rule. Under these conditions, tolerance increased somewhat, and subordinates got some food before they were driven away. Moreover, the pecks were usually small—i.e., they were quick interruptions in the feeding of the dominants—and they elicited small retreats from the subordinates. All reverse and inconclusive pecks were of this nature, and very often pecks in accordance with the pecking order occurred on the same day and sometimes immediately before or after. The pecks back and forth did not appear to be connected events within a hostile sequence. The reverse peck always caused the dominant to retreat, with a single exception: R pecked O and was completely ignored. R immediately left the area.

Unstereotyped sequences of behavior.—The behavior of the junco is capable of a considerable amount of variability in combining various patterns. (*a*) LR and R were on the ground. In a long, mutual hostile display, LR circled about R at a distance of about a foot, while R pivoted to face LR. Both birds stretched up and lifted their heads, but no peck was delivered. (*b*) CW began to eat on the bench, 3 feet from LR. (Ordinarily, two birds would eat and ignore each other at that distance.) LR at once flew into the hedge, crept slowly through it, and perched at a point 8 inches above CW. The latter then stopped eating and went into the hedge, whereupon LR flew back to its original position and began to eat. Again CW alighted 3 feet away, and the whole procedure was gone through twice more. There was no pecking or display of hostility. (*c*) LR tolerated R for about 2 minutes, then both suddenly flew into the hedge together and perched. LR then crept slowly through the hedge until it was on the same branch as R and 3 inches above R. The latter then began to hop down the branch away from LR,

with hostile posturing, moving about an inch at each hop. LR followed closely. They then hopped to the bench and ate together again. The whole sequence took 11 minutes. (*d*) In a somewhat similar manner RY forced BR down a branch by very small pecks responded to by very small hops. (*e*) LR was eating at the only available tray while OC perched above and appeared to watch LR. LR left the feeding bench and went into the hedge, while OC immediately took its place on the bench. LR returned at once, and OC left at once. This proceeding was gone through five times. The last time terminated in OC's remaining when LR returned. OC was then tolerated by LR, and they ate together. No peck was delivered in this sequence. (*f*) It was characteristically the habit of the birds to perch in the hedge before eating, move their heads to and fro, and present the appearance of looking at the various parts of the feeding area to decide where to eat. When dominant birds did this, they usually cleared a place very quickly. A subordinate might remain perched and wait, or it might use one of the patterns of avoidance described above or a pattern likely to gain tolerance. (*g*) Some of the birds showed individual tendencies to follow one sequence more often than another. O, for example, finding RY or tree sparrows on the bench, usually foraged on the ground. CW often crept through the hedge close to the bench and hopped on it tentatively. YG often flew back and forth between the hedge and the ground when it was unable to eat. SO sought for food around the trap. As was said above, R and YG attempted frequently to eat with dominant birds.

CHART III

JANUARY 10 - MARCH 11, 1948

TREE SPARROWS

	E	L	Y	S	G	N
E		72	47	51	3	4
L			51	11	2	4
Y				36	16	8
S			3[x]			4
G						1
N						

ANALYSIS OF CHART III

Regular pair-relations	13
Regular pair-relations, including reverse pecks	1
Complete blanks	1
Possible pair-relations of six birds	15

DOMINANCE IN THE TREE SPARROW FLOCK

Of the six birds in this flock, Y was the only bird banded by Westfall in 1947. Though adult, Y was an extremely small bird. Chart III gives the data on dominance among the tree sparrows.

The chart indicates a linear hierarchy uncomplicated by triangular pecking. Variation in the frequency of contacts is largely due to the varying number of visits to the station. E, L, and Y visited the station for 2 months, S for 1 month, G and N for 10 and 9 days, respectively.

BEHAVIOR PATTERNS ASSOCIATED WITH DOMINANCE IN THE TREE SPARROWS

The following patterns were observed: (1) All the patterns of avoidance described for the juncos were observed among the tree sparrows also. (2) Twelve

instances of indifferent tolerance were observed, L being the tolerating dominant in ten cases and E in two. As with the juncos, the beta bird was more tolerant than the alpha. (3) Three instances were observed in which a subordinate approached a dominant with hostile posturing, apparently making a "request" for tolerance. In two of these the hostile subordinate was promptly pecked and retreated. In the third, a case in which the food was concentrated on one tray, L approached E with hostility, resisted E's peck, and a fight of maximum intensity ensued. The two birds then ate together, 6 inches apart. It should be noted that this pattern of behavior was identical with that observed in two of the fights between juncos, and that the victory of L did not alter the pecking order, since E displaced L as usual on the following day. (4) The pattern of dominant behavior displayed by E, the alpha bird, was similar to that of the dominant birds in the junco flock. The behavior of the subordinate tree sparrows relative to the dominant was somewhat different. They were more persistent than the juncos in snatching seeds and in returning after being driven away. On one occasion E drove L away eleven times and S twelve times within 9 minutes. (5) The pattern of reverse pecking was similar to that of the juncos. (6) Sequences of unstereotyped behavior such as were described above in the case of the juncos did not occur among the tree sparrows.

DOMINANCE IN THE INTERSPECIFIC RELATIONS OF JUNCOS AND TREE SPARROWS

It was the habit of the juncos and tree sparrows to visit the station together as well as separately, and they did so in all possible combinations, ranging from members of one species accompanied by a single individual of the other to groups composed about equally of the two species. When mixed, they behaved like a single flock, showing dominance, tolerance, and avoidance in their mutual relations. But there was an anomalous feature, first noted in 1947, in these relations. In 1947 a triangle was observed between LR, YC, and a visiting tree sparrow. Chart I shows LR to be dominant to YC. Three times LR retreated before the tree sparrow, and three times YC drove the tree sparrow away, though YC was low in the hierarchy and was, in addition, a crippled bird unable to fly. Because long sequences of such triangles were observed in 1948, observations were undertaken on the relations of dominance between birds of the two species. As an illustration: YG (the omega junco) was driven from the feeding tray by RY (the alpha junco); RY was pecked off by E (the alpha tree sparrow); then E was displaced by YG. It became clear that, for the most part, the four dominant juncos were subordinate to the tree sparrows, while, on the contrary, the six[4] subordinate juncos were dominant to them.

Explanation of Charts IV and V.—It is not intended to arrange the birds of the two species in a linear hierarchy. Charts IV and V are merely convenient devices for showing relations between the three groups: the four high-ranking juncos, the tree sparrows, and the six low-ranking juncos. The asterisk and the triangle used as symbols have the same numerical significance as in Charts I, II, and III.

In Chart IV the birds are arranged on the presumption that the tree sparrows dominate the four leading juncos. The figures below the diagonal show all the pecks that are contrary to that presumption.

All events recorded in this chart were pecks; there were no fights. Four of the blanks were in the records of G and N, the tree sparrows that had few contacts

[4] NR had no contacts with the tree sparrows.

because of the shortness of their record with the flock. In fifteen out of twenty-four possible relations the tree sparrows were dominant, with E (the alpha tree sparrow) most conclusively so.

ANALYSIS OF CHART IV

Pair-relations that show tree sparrows as dominant	13
Pair-relations that show tree sparrows as dominant with reverse pecks	2
Pair-relations that show juncos dominant	2
Single inconclusive pecks	2
Complete blanks	5
Possible relations of groups of four and six birds	24

In Chart V the birds are arranged on the presumption that the six low-ranking juncos dominate the tree sparrows. In this chart there is a new type of inconclusive pair-relation, viz., balanced relationships in which there are several contacts in favor of the presumption of the chart and about an equal number against it.

Of the nine blanks, three were caused by the fact that junco RL left the station before S, G, and N arrived.

The reverse pecks in Charts IV and V were delivered under the conditions of tension described above under "Reverse and Inconclusive Pecks." The reverse pecks delivered by E to YG (Chart V)

CHART IV

JANUARY 10 - MARCH 11, 1948

	TREE SPARROWS						DOMINANT JUNCOS			
	E	L	Y	S	G	N	RY	O	LR	CW
E							11	9	8	7
L							6	1	11	4
Y							3Δ	1	7	1Δ
S							1		1	
G							4			1
N									2	
RY			7Δ			1				
O										
LR			1✕			1✕				
CW			2Δ	1						

require additional comment. YG, the omega junco, had been consistently dominant to E, the alpha tree sparrow. On the occasion of the first reverse peck, YG was clearly not well. This was shown by a violent shudder when a house sparrow alighted clumsily near YG, by YG's extreme nervousness while eating, and by its failure to notice and peck tree sparrows Y and S. Suddenly E made a violent run at YG, with spread wings and fanned tail, and YG retreated. Two days later YG again retreated before E upon a second contact and showed hostility without retreating upon a third. A week later, upon a fourth contact, YG pecked E as usual.

In two respects relations between the birds of distinct species differed from those between birds of the same species. First, there were five cases in which two reverse pecks occurred within a pair-

ANALYSIS OF CHART V

Pair-relations that show juncos as dominant	11
Pair-relations that show juncos as dominant with reverse pecks	10
Pair-relations that show tree sparrows as dominant	2
Pair-relations that show a balance of dominance	4
Complete blanks	9
Possible pair-relations of two groups of six birds each	36

CHART V

JANUARY 10-MARCH 11, 1948

	SUBORDINATE JUNCOS						TREE SPARROWS					
	OC	R	RL	SO	BR	YG	E	L	Y	S	G	N
OC							10	8	11	1	Δ	
R							8	21	37	2	1	3
RL							3		3			
SO							6	4	9	4		
BR							6	6	27	1	2	
YG							19	7	50	11	Δ	
E	2X	2X		8	6	2X						
L	1X	6		1X	2X	1X						
Y	2X	1X			1X							
S												
G	6Δ				2	4Δ						
N												

relation. Second, there was much back-and-forth fighting as well as pecking in three pair-relations: junco R and tree sparrow L, junco SO and tree sparrow E, junco BR and tree sparrow E. These pair-relations were sufficiently balanced to preclude any decision as to which bird was dominant. The relation between R and L does not appear to be balanced in the chart, since R was dominant to L twenty-one times and L to R only six times. But R pecked L fourteen times in their early contacts, after which the relation settled down to be one of balanced pecking and fighting.

Interspecific tolerance was common, both of the indifferent and the hostile type. Some of the fighting may have resulted from an approach for hostile tolerance, but the preliminary behavior was not clear enough to make the point certain.

DISCUSSION

IRREGULAR CONTACTS IN CHARTS I, II, AND III

There is no evidence that reverse pecks signified instability in the pecking order. Inspection of Charts II and III shows that reverse pecks occurred in cases where the regular pair-relation was established by a substantial number of contacts. Furthermore, the contacts which were in agreement with the pecking order were made both before and after the reverse pecks. These reverse pecks uniformly occurred at times of tension and rapid eating, and this fact may have played a part in explaining their occurrence. Such a condition may give rise to inattention on the part of a dominant bird, and this may operate like a temporary weakness to give the subordinate an opportunity momentarily to display dominant behavior. It has frequently been noted that subordinate members of a social group are quick to sense the weakness of a dominant (Pardi, 1948; Diebschlag, 1940; Lorenz, 1935) and to assume a dominant role in consequence. The temporary dominance of tree sparrow E to junco YG is a case in point.

There are several instances in Charts I and II in which a single irregular peck is the only contact between two birds. Such cases were classified as inconclusive because there were two possibilities. Further pair-contacts, if regular, would have shown them to be reverse pecks or, if irregular, would have shown them to be triangles. There was only one stable triangle in Chart II as against six reverse pecks. This fact and the further fact that inconclusive pecks, like reverse pecks, occurred only under the special conditions described in the preceding paragraph, make it appear likely that the pecks classified as inconclusive would have turned out to be reverse pecks oftener than triangles.

INTERSPECIFIC RELATIONS

The principal aggregations consisting of more than one species take the form of common roosts, migratory flocks, and feeding flocks (Friedmann, 1936). Darling (1938) believes that certain elements of behavior in two species of colonial-nesting gulls which nest in close propinquity have biological value in stimulating breeding activities. Lorenz (1935) remarks that birds respond, with beneficial results, to the warning notes uttered by other species in their biotope. Friedmann (1936) has suggested a biological origin of mixed flocks seeking the same food, but otherwise he attributes the origin of such flocks to the "general gregariousness of most small birds."

Presumably there is a gregarious drive released by visual stimuli from other birds. This factor, added to co-occupation of a habitat and similar responses to weather conditions,[5] might sufficiently

[5] The writer's findings about simultaneous responses of several species agree with those of Van De-

account for general gregariousness in feeding flocks. Similar size and similar habits of eating also encourage such aggregations.

The information conveyed in Charts IV and V, with the accompanying descriptions of the behavior of the mixed flocks, however, suggests a closer integration than is implied by mere gregariousness. In the first place, apparently the two species have real social relations based on mutual individual recognition. In the second place, there seems to be a pattern in these relations, although that pattern is unusual and unexpected.

With reference to the first point, it seems probable that an interspecific social relationship, as distinct from mere aggregation, must depend upon the presence of an additional link in the chain of symbiotic physiological and psychological factors that give rise to winter assemblies. In these assemblies, species are often seen in proximity which largely ignore or avoid each other. The additional link, in the case of the two species under discussion, is presumably the near-identity of the components of their social behavior. Pecking, threatening, fighting, and mutual tolerance—the standard forms of social contact—are exhibited by the juncos and the tree sparrows with like postures and in like intensity. The analyses of Charts IV and V indicate that the interspecific pair-contacts gave rise to pair-relations which were stable in a large majority of cases.

Of the contacts that are not stable and that might be said to show peck-dominance rather than peck-right, three (L-R, E-SO, E-BR) were fairly well balanced, and these were differentiated from other pair-relations by considerable fighting. There was no discernible factor, such as territoriality, to account for peck-dominance. In respect to the five cases in Chart V in which there were two reverse pecks rather than one, it is impossible to say whether or not these should be taken as similar to other reverse pecks and therefore as due probably to the inattention of the dominant bird. They nearly all occurred under the crowded conditions in which reverse pecks occur. If this explanation were accepted, it would presumably mean that the tree sparrows, when subordinate to juncos, are quicker to sense an opportunity for reverse pecking than they are among themselves. It is of course possible that these are cases of peck-dominance depending on some factor less casual and transitory than the inattention of the dominant bird—a factor which the observer was unable to discover.

The second main point of interest is that the pair-relations fell into a pattern. A somewhat similar case of social organization in a mixed flock of geese has been reported by Jenkins (1944). Not only were there stable pair-relations but the relations were not random. A family of blue geese was dominant both to the members of a group of Canada geese and to another pair of blue geese. The Canada geese, however, were dominant to the last-mentioned pair of blue geese. The arrangement was thus hierarchical, but the Canada geese divided the total group of six blue geese, being subordinate to four dominant blue geese but dominant to the subordinate pair. One species was thus not dominant over the other.

The tree sparrow–junco complex exhibits a still more unexpected pattern. The juncos, like the blue geese, are divided into two groups by a group of a different species, but the three groups do not form a hierarchy, since the four domi-

venter (1936), with the exception that cloudy weather with precipitation ranks first rather than second as a stimulus to activity. The two sets of observations agree about the depressing effect of uniform cloudiness without precipitation.

nant juncos are, in the main, subordinate to the tree sparrows, while the six lower juncos are, with the exception of the irregularities previously noted, dominant to the tree sparrows. The system is therefore one of many triangles.

If we accept this pattern provisionally and attempt an explanation, it is clear that some factor must exist that differentiates the four upper from the six lower juncos. The only information available relative to the sex and age of the birds is that one of the upper group (O) is known to be male and two (LR and CW) are known to be adults, and of the lower group one (SO) is known to be male. The conjecture that adult male juncos are subordinate to tree sparrows, while young males and females are dominant to them, would be as unexpected as the situation to be explained.

The question naturally arises at this point as to whether observations of one mixed flock during one season are sufficient to be trustworthy. Three comments may be made on the possible effects of further data. (1) There are at least three patterns of organization that may be regarded as antecedently more probable than the system of triangles indicated by the data: (*a*) a mixed linear hierarchy; (*b*) dominance of one species over the other; and (*c*) a hierarchy of three groups like the series reported by Jenkins. The data already collected precludes any of these patterns. (2) It is possible that the system is one of peck-dominance. In that case the outcome of more numerous observations might include the present data and might strengthen, modify, or reverse the present indications. (3) It is conceivable, though perhaps not probable, that the interspecific relations in the flock studied were accidentally misleading in suggesting any pattern. In other words, the outcome of a sufficient run of observations on different flocks might show that the pair-relations are really random. A reasonable conclusion may be that authentic interspecific social relations will probably fall into some pattern, and that the tendency in the observations is too marked to be summarily dismissed but too improbable to be accepted. More data are therefore required.

HOSTILE TOLERANCE AND FIGHTING

The threatening posture with which a subordinate junco sometimes approached a dominant appeared to have a compulsive effect on the dominant to respond with the same erect, head-raising gestures. Some dominants were more vulnerable than others to this kind of stimulus. The subordinate bird R, on the other hand, seemed to be under compulsion to use this technique of hostile approach. R would hop into the feeding tray with a dominant even when food was available in the next tray, as if stimulated by the sight of the bird eating rather than by hunger or the sight of food.

The conclusion seems sound that fighting in the 1948 flock of juncos and tree sparrows had two functions—to place the birds in the hierarchy according to their aggressiveness and to enable a subordinate to gain tolerance for eating. The interpretation of a fight in one or the other of these two ways depended upon its origin, and frequently this could not be observed. The fights of CL (the bird not entered in Chart II) seemed to be aggressive attacks such as might be expected to place the bird in the hierarchy. Possibly the large amount of inconclusive fighting was due to "requests" for tolerance rather than to aggressive attacks by subordinate birds. It seems noteworthy in this connection that none of the fights observed altered the pecking order.

The somewhat unsettled appearance

of the junco flock in January, 1948, turned out upon analysis to be largely due to the behavior of a single bird, R, whose turbulent behavior with OC may be reviewed as one example. There were many instances of hostile tolerance, two pecks successfully resisted by R and four fights. Yet, throughout it all, OC pecked R twenty-one times, and R's position in the hierarchy was the same at the end of February as at the beginning of January.

DISTRIBUTION OF PECKS

Observers of animal societies have stated that a linear order of dominance in a free society differs from the same type of organization in a captive flock (Lorenz, 1935; Pardi, 1948). One difference is said to lie in the greater stability of the free society. Another lies in the distribution of contacts between dominants and subordinates and in certain consequences that flow from this distribution. The distribution of contacts arises from three facts: the high-ranked individuals have more contacts, the greatest number of contacts for any individual occurs with its next-ranked subordinate, and the number of contacts between any two individuals diminishes the more remote they are from one another in the ranking. Thus a "theoretically perfect" chart for a linear order in conformity with these standards would show (1) diminishing numbers in each horizontal line from left to right; (2) a smaller number at the left of each line compared with the number at the left of the line above; and, in consequence, (3) a line of diminishing numbers along the diagonal from upper left to lower right. The result would be that the low-ranked individuals, though dominated by more superiors, would receive fewer pecks than would higher-ranked individuals, and the well-known "persecution" of the omega individual would not occur. Obviously, a chart of this sort depends on the assumption that observations of contacts between any two individuals are equally probable, an assumption perhaps approximately realized in observations of a wasp's nest or a colony of jackdaws.

So far as concerns the stability of a free society, the behavior of both the juncos and the tree sparrows confirms the expectation that a free society will prove to be stable. In respect to observing contacts between members of a free flock, however, it is practically impossible to meet the condition implied in the theoretical chart, since the observer has no power to equalize the number of visits of the several birds to the feeding station.

In the case of the tree sparrows the distribution of contacts between dominants and subordinates in Chart III conforms well to the theoretical pattern. It should be noted, however, that in this case the approximation is helped out by the short records of S, G, and N. Chart I is too incomplete to make comparison with a standard chart significant. Chart II does not conform even approximately to pattern, because the frequency of contact between two birds varies according to the frequency with which the birds visited the station. If some weighting is done to offset these differences of frequency, there is in some cases an approximation to the expectation that any individual will have the largest number of contacts with its next-ranked subordinate. Thus the frequency with which RY pecks O (Chart II) is high, considering that O was an infrequent visitor. The same is true of O's relation to LR. In fact, O's pecking record is a reasonable approximation to pattern. CW's relation to OC is in accord with expectations, without weighting.

But even if discrepancies due to unequal numbers of visits to the station could be eliminated, it seems doubtful

whether the juncos would approximate satisfactorily to the theoretical pattern described. The record of YG, the omega junco, in successfully resisting pecks from O and LR, suggests favoritism. The numerous pecks given to YG by R and BR might also indicate antagonism. The unstereotyped sequences of behavior, which seemed in a sense to "play" with the pecking order, and the records of R and YG suggest that the junco has a considerable capacity for individualized behavior. Possibly this capacity is related to the variability of the junco in its hereditary characteristics. Miller (1941, p. 173) remarks: "Among birds, the genus junco is especially rich in instances of natural interbreeding of well marked forms and it shows great variety in degree and kind of differentiation."

DOMINANCE AND TERRITORY

The complicated intermingling of dominance and territory as related to aggressive behavior has been frequently discussed (Tinbergen, 1936; Allee, 1942; Jenkins, 1944). In a brief general comment Lorenz (1935) remarks that there seem to be two principles—that of the hierarchy, which is the organizing factor in certain social species, and that of individual territory, which seems to prevail in certain other species. Lorenz supposes, however, that in many species in which one of these principles is developed the other is latent. The latent capacity for either kind of organization, he supposes, may be brought out under experimental conditions. As illustrations he uses the dominance of solitary caged birds over the introduced stranger and the pecking order among captive unsocial birds. Collias (1944) calls attention to latent territoriality when he remarks that, though hens are not supposed to be territorial birds, the production of eggs by low-ranked pullets can be increased by placing these birds in individual egg-laying cages. Other experiments (Noble, Wurm, and Schmidt, 1938) indicate that a group of strongly territorial birds crowded into a strange neutral area will develop a hierarchy.

A further conclusion by Lorenz is that one or the other of the two principles of organization—the successful defense of individual territory or the peck-right hierarchy—will prevail in a species. Diebschlag (1940) believes that in the case of the house dove successful defense of individual territory is bound to result in occasional successful attacks by the weaker bird and that the resulting peck-dominance does not conceal an underlying peck-right. He seems rather to suppose that the permanent drive in this species to mark off and defend an individual spatial refuge precludes the coexistence of the classical peck-right hierarchy.

The results obtained by Bennett (1939, 1940, 1942), so far as the question of hierarchy and territoriality are concerned, were somewhat different. Working with nonbreeding, homosexual flocks of ring doves in order to eliminate the territoriality peculiar to the breeding cycle, Bennett found that the relations of the birds were peck-dominant but that the peck-dominant relations fell into hierarchical form. Low-ranked birds did not normally locate and defend territory. When made aggressive by injections of testosterone, however, they started territorial activities, and, at the same time, pair-relations determined by peck-right became more numerous within the flock.

The observations of free-living birds in this paper indicate that both hierarchy and individual breeding territory have a place in the social life of juncos and tree sparrows, but at different periods of their life-cycle. There is also some evidence

that common territory functions along with hierarchy in the winter flock of juncos (Westfall, 1947), and there is strong evidence that this is true of tree sparrows (Heydweiller, 1935). The theory of common territory is a possible explanation of the dominance of resident juncos over the migrant strangers in April, since it would make it possible to assume a general accession of aggressiveness because of territoriality. Even the lowest members of the resident flock were dominant to migrants. Comparable observations on tree sparrows are not available, but chickadees show similar behavior (Odum, 1941). The fact that juncos and tree sparrows hold individual territory in their breeding period and common territory in the winter may be the reason for the definiteness of their winter organization, as compared with the fluctuating relations found in caged flocks of paired canaries (Shoemaker, 1939). It should be noted, however, that the observations in the present paper concern only the feeding phase of social activity. The common roost, known to be used by winter flocks of juncos (Whittle, 1926), is an additional center of group relations and a point at which territoriality might, by analogy, be conceived to exist (Kalmbach, 1932).

THE ATTAINMENT OF ORGANIZATION

Several questions naturally suggested by laboratory studies of flocks of hens may be considered, though they cannot be answered with satisfactory completeness. (1) Was the initial contact of decisive importance? (2) What was the behavior of members of the flock to strangers? (3) What was the behavior of strangers on joining the flock? (4) Was there any evidence of the imperfect assimilation of members of the flock? The remarks below refer only to the juncos.

(1) The observer had no way of knowing whether a first observed contact was actually an initial contact. The birds naturally might have had previous contacts in the woods outside the range of observation. (2) Members of the flocks showed no reaction to presumably strange birds analogous to the chasing of the strange hen newly introduced into an organized flock (Douglis, 1948). (3) The strangers fell into three classes. The subordinateness of the migrant strangers has been mentioned. The casual visiting strangers seemed to have positions in the flock. This fact was puzzling to the observer in 1947 because the visitors were assumed to be strangers like the spring migrants and yet they had varying positions in the flock. The interesting experiments of Douglis (1948) suggest that members of the flocks of juncos became acquainted on the edges of their territories, so that actually they were "assimilated" into the flocks they visited. The late joiners formed another group that may have been strangers. Two birds, RL and BR, on joining the flock late, showed reactions analogous to those of strange unaggressive hens by staying away from the food and avoiding contacts, while a third, CL, showed the reactions of the aggressive hen by making contacts promptly with the three highest birds of the flock. (4) The behavior of the four intermediate birds in 1947 (Chart I) might be regarded as evidence of imperfect assimilation. They seemed to be self-isolated by avoidance.

In conclusion the question might be asked whether the wild birds were unorganized or disorganized when they occupied their winter territory after migration. Since laboratory birds are often without social relations at the start and are said to "compete on the individual level," it is easy to carry this concept over to a wild flock and to assume that a winter population starts as an aggregation without organization. This assump-

tion, however, has been questioned. Darling (1938) remarks that nothing is known about the "unifying element" in fall and winter flocks. Whittle (1926) raised the question of whether groups may not be formed during the autumn flocking which persist through migration. Heydweiller (1935), who marked nesting tree sparrows with colored feathers in Churchill, Manitoba, describes how easily, on a certain day in August, the family unity and territory broke down and suddenly young and old birds were eating together on each other's grounds. Wider wandering in small changing groups soon followed, and the marked birds were lost to view. There is some evidence that both tree sparrows and juncos travel in groups during migration (Heydweiller, 1935; Whittle and Fletcher, 1924), and there is no doubt that individuals frequently return to the precise localities where they had previously wintered. In the absence of evidence to the contrary, it may be reasonable to suppose that these species pass easily and naturally from one phase of social organization to another in the course of their yearly cycle and are at all times embraced within some kind of social group. Of the winter feeding flocks of both species it may be said that they seemed to move and have their being within the influence of the pecking order.

SUMMARY

1. Two wild flocks of wintering juncos observed in successive seasons were socially organized in a straight-line pecking order, modified by triangular relations and reverse pecks.

2. A wild flock of tree sparrows feeding with the juncos was socially organized in a straight-line pecking order, modified by reverse pecks in one pair-relationship.

3. The juncos had repeated patterns of behavior exemplifying avoidance, tolerance, and dominance.

4. The juncos also displayed unrepeated, individualized sequences of behavior, linking avoidance, tolerance, and dominance in unstereotyped ways.

5. The tree sparrows showed avoidance, tolerance, and dominance by approximately the same methods as did the juncos, but their behavior fell into stereotyped patterns.

6. In each species fights originated in a special approach for tolerance by a subordinate bird, the result being, if the fight were successful, that the subordinate ate with the dominant.

7. All intra-specific pecking orders were stable, showing no change in social status.

8. The two species were compatible and had an interspecific order of dominance in which the four dominant juncos were subordinate, as a rule, to the tree sparrows, while the latter were, or tended to be, subordinate to the six subordinate juncos.

9. Irregular contacts and fights were more numerous in interspecific than in intra-specific relations.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

ORGANIC FORM AND RELATED BIOLOGICAL PROBLEMS[1] BY SAMUEL J. HOLMES

Reviewed by D. P. COSTELLO
University of North Carolina

THIS book is a collection of thirteen essays, six of which deal with the problem of organic form. These six have a stronger thread of continuity than have the seven remaining essays. Chapters ii through vi were previously published in the *Scientific Monthly* for June, 1942, and for September through November, 1944. Chapter xiii, "Recapitulation and Its Supposed Causes," appeared in the *Quarterly Review of Biology* for December, 1944.

The early part of Holmes's book deals with the more fundamental problems of embryology or with what probably may be considered the more significant problems of all biology. He discusses integrative levels, mechanism and vitalism, preformation and epigenesis, mosaic versus regulative development, developmental adaptation, divergent differentiation, symbiosis, division of labor among cells and individuals, and various aspects of regeneration. From the philosophic viewpoint, the author considers whether the biologist can offer any satisfactory explanation as to how living organisms initially form and are constantly being remade, regenerate cells or lost organs, maintain the relative proportions of their parts, and preserve the normal balance of their physiological functions.

The later part of the book deals with such diverse topics as the reversal of development (reduction through dedifferentiation, starvation, etc.), the regeneration of the blood, the roles of gradients and genes, cancer as a biological problem, adaptation, basic life-functions, the theory of recapitulation, and the bearing of recent work dealing with autocatalytic enzymes and viruses on the old problem of the origin of life.

[1] Berkeley and Los Angeles: University of California Press, 1947. Pp. v+169. $5.00.

The essays are well written, as compared with the usual personal philosophies of science. Many entertaining analogies are drawn between biological explanations and classical Greek myths. The author's analyses attempt to present well-rounded and formal explanations of the problems in terms of present knowledge, giving the impression that in many cases the problems are essentially solved. Actually, many of these problems are far from solved, even at the biological level, to say nothing of their physicochemical basis. Generalizations in biology are of little significance unless they can be used for prediction of future events, and hypotheses are useless unless they suggest new modes of attack, aiming toward the ultimate solution of the problems.

While dealing with some of the same general problems, this book stands in striking contrast to D'Arcy W. Thompson's *On Growth and Form*. The former is philosophical, the latter, physicomathematical. It is perhaps the contrast between the attitude of one who wishes to have things orderly and settled in his own mind and the enthusiastic fervor of one who adds disorder by opening up new fields of scientific speculation.

In the chapter on the regeneration of the blood and its bearing on the morphogenic theory, the author points out the inadequacy of the "field or gradient theory" in accounting for cases of regeneration which do not involve restoration to a fixed physical form. Holmes concludes that, in the restoration of blood plasma and corpuscles from separate sources to replace a lost volume of blood, anything basically analogous to the regeneration of a crystal is inconceivable.

The chapter on "Genes versus Gradients" has such statements as ". . . the direction in which parts differentiate . . . is caused by specific potencies that are called into action and by

the relative rates at which they act. The potencies, or genes that speed ahead of others, are those that lead to organs of greater size or structural complexity." In a long discussion of Child's gradient theory, Holmes apparently fails to realize that if all the nuclei (and their genes) in all the cleavage blastomeres are alike, then the differences in the prospective significance of these blastomeres must be due to cytoplasmic differences. There is much experimental evidence for Child's contention that genes must be relegated to a secondary role and that early embryonic determination is under cytoplasmic control.

Direct references to the literature, as used in technical scientific articles, are not given, and the Bibliography, which emphasizes books and review articles, is inadequate to document properly the facts presented. The author, realizing this, has suggested that the reader who wishes to pursue the subject further may consult the bibliographies of the review articles. This fact, as much as the general style, indicates that Holmes is directing his work to an audience of educated, philosophically minded laymen or to scientists in allied fields rather than to professional biologists. As such, it is a worthy attempt to elucidate the status of some of the fundamental problems of biology. We do not have many semipopular books on the philosophy of biology. These days, almost every educated man knows something about nuclear atomic structure, galaxies, the speed of light, and the structure of our universe, but few are acquainted with the problems of growth, differentiation, and regeneration.

THE USE OF ISOTOPES IN BIOLOGY AND MEDICINE.[1] BY CLARKE, UREY, SEABORG, AEBERSOLD, NIER, CORYELL, KAMEN, MELVILLE, SPRINSON, WOOD, BLOCH, GREENBERG, CHAIKOFF, HAMILTON, HALL, HERTZ, BALE, NICKSON, DANIELS

Reviewed by WILLIAM R. DURYEE
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ALTHOUGH zoölogy has not yet felt the full impact of nuclear physics, radioactive isotopes have now been used for more than twenty-five years in certain areas of biological research. Detailed reference books, such as the present one, are therefore urgently required. If a debate were undertaken on the question of whether a reference volume is more useful when prepared by an individual expert or by a group of experts, this collection of papers from the symposium held last year at the University of Wisconsin would support the latter alternative. It further has the advantage of being early in a field of ever growing scientific importance. But it is possible that the title is a misnomer, in not replacing the word "biology" with the designation "biochemistry." The illustrations are clear, although few in number. Numerous errata have been excused by the publisher on the grounds of rapid publication.

It would be unjust to the distinguished collaborators of this volume to criticize their efforts from a negative point of view, because here is a dependable, informative, and readable presentation of selected phases of the isotope work through 1947. Dr. Urey's and Dr. Seaborg's discussion of the separation and preparation of isotopes will be of less immediate use to biologists. On the other hand, Aebersold's summary of the availability of more useful radioactive atoms is especially worth while for those contemplating the use of tracers. Equally valuable is Coryell's fundamental analysis of instrumentation. It is not generally realized by biologists that much significant work can be done with an inexpensive Lauritsen electroscope instead of the more temperamental Geiger-Müller counters, with their accompanying amplifiers and scalers.

Few zoölogists, even those trained in physiology, are expected to undertake synthesis of radioactive compounds for their own work. Consequently, reliance must be placed on chemists and institutions specializing in this type of work. It is likely that the chapter on preparation of compounds will be of theoretical, rather than of practical, interest.

Turning to the latter half of the volume, the reader will find excellent introductory discussions of tracer studies on metabolism of proteins, carbohydrates, and lipids. It is now well established that tissue proteins and their constituent amino acids, as well as fats and carbohydrates, are in "a state of ceaseless movement and renewal." The introduction to the literature of the past decade alone commends the book to

[1] Madison: University of Wisconsin Press, 1948. Pp. x+445. $5.00.

biologists generally. Not without value is the partial repetition and re-emphasis from different points of view of the role of P^{32}, heavy hydrogen, and the three carbon isotopes. More detailed expositions of atomic exchange and molecular synthesis can be found elsewhere in the literature, but no other single volume to date has given such a broadly balanced interpretation.

The chapter by D. M. Greenberg on tracer studies of mineral-element metabolism appears to this reviewer to be the best of the volume. His conclusions on bone metabolism in fractures, on iron catalysis, and on carbohydrate turnover in muscle and liver with P^{32} are well presented. It forms an essential introduction to later studies on C^{14} metabolism of proteins that readers will find elsewhere. Other chapters on iodine metabolism in thyroid tissue, medical applications of tracers and radiophosphorus in blood diseases, while informative, are not sufficiently precise to permit "quantitative evaluations," as occasionally admitted by the authors. While the book contains no systematic review of ion-exchange dynamics, there is sufficient material to give the reader a detailed understanding of the problems involved.

Not to be overlooked is an excellent discussion of the biological effects of radioactive materials within tissues. Under the somewhat misleading chapter heading of "Health Hazards," W. F. Bale has brought together an instructive series of sample calculations of energy outputs from different emitters. These calculations of radiation dosages, in roentgen equivalents based on physical constants readily found in tabular data, are of front-rank importance for biologists not trained in physics and are not easily found elsewhere.

Returning to the volume and its significance for physiological zoölogists, one cannot help remarking that none of the contributors is himself a zoölogist. If the reader runs back over copies of *Physiological Zoölogy* for the last two years, he may ask the question: How many of the contributors could have utilized radioactive isotopes in their research? Without attempting a specific answer, it is apparent at once that a goodly number of zoölogical and botanical problems could be attacked with the aid of tracers. For their own research in the next decade few zoölogists or botanists can longer ignore the possibilities of radioisotope techniques, either simple, varied, or even bizarre as such might now appear. For example, the development of good radioactive "tags" for fish and other aquatic animals is needed. Such speculations in planning are not without value for future work. A distinguished scholar some years ago pointed out that scientists generally and biologists in particular are inclined to think too much of what has been done and too little of what can be done. The collaborators in this volume have reviewed "the developments in atomic physics that are especially applicable in the fields of biology and medicine."

However, at this early date it would be rash indeed to forecast the exact extent of isotopic research in the fields of zoölogy. Too few are trained in the new techniques. Another limitation is that traced elements, tagged compounds, and internal radiations are only tools. It is important today to realize that most of the best research of tomorrow must still be done without help from radioactivity. The results so beautifully presented in this volume should stand both as an encouragement and as a warning. As isotopes come to be used more and more as *tools* of basic research, physiology will benefit. On the other hand, if isotopic research becomes haphazard or too specialized, the remainder of biology may suffer. It is expected that this symposium will point toward closer co-operation among scientists, particularly in the area of biophysics.

The reader will find much to stimulate his thoughts on the question of ion exchange and on general implications of tracer work in physiological reactions. But, in spite of the deeply significant results reported, he may feel that the classical applications of tracers to the field of zoölogy (in Romer's sense) still remain for a vigorous and imaginative future. While some biologists have considered that isotopic work has produced much fundamental new knowledge of the organism as a whole, there are others who believe that the strictly biological problems of greatest importance do not lie in the domain of intermediary metabolism. This volume paradoxically may serve to reinforce the latter belief.